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Induction by Regions lateral to the Streak in the Chick Embryo

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WITH TWO PLATES

THE discovery of the organization centre of Amphibia by Spemann (1918) opened a new field in the outlook of experimental embryology; but there were several technical difficulties to be overcome in the application of the concept of the organizer to avian embryology. In this connexion the tissue culture technique has several advantages over the earlier methods of *in situ* sectioning or chorio-allantoic techniques, and it has helped a great deal in the understanding of the processes of development in the chick.

It has already been proved that at least the anterior third of the primitive streak has an organizing function (Waddington, 1932); but regions outside the primitive streak have not been systematically tested so far for the inducing capacity. In the present studies an attempt is made to study the extent of the organization centre in the chick embryo at the definitive primitive streak stage.

MATERIALS AND METHODS

Fertilized fresh eggs, obtained from the Poultry Research Centre in Edinburgh, were incubated at 37·5° C., to the desired stage of development.

The embryos were cultivated *in vitro* by the technique described by New (1955). After about 22–24 hours of culturing, some of the entire specimens were fixed in Bouin and stained with dilute Delafield's haematoxylin; they were then cleared in cedar wood oil and microphotographed. Sections were serially cut at 10 μ. They were stained in Delafield's haematoxylin. In all, 303 grafts were made and 270 examined histologically.

The experiments consisted in cutting a single blastoderm of a definitive primitive streak stage of development (the length of the streak varied from 1·7 to 2·1 mm.) into a certain number of median and lateral squares each of about 0·3 mm. side. Tungsten needles were used for cutting the blastoderm, and the measurements were made with an ocular micrometer.

The position of the cuts may be ascertained from Text-fig. 1. The primitive

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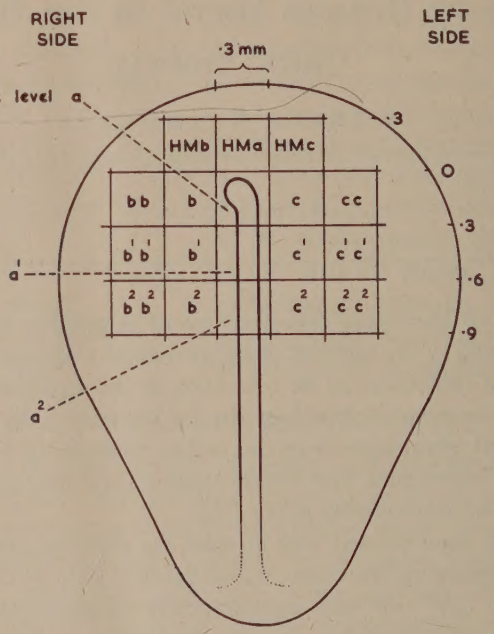
streak was cut into units of 0·3 mm. square; the first cut was made to include the node level of the streak (*a*) and the subsequent cuts were made with it as an origin. The cuts containing the parts of the streak at different levels are labelled



TEXT-FIG. 1. Scale drawn map of a primitive streak stage blastoderm (ventral view) showing the scheme of grafting made in the experiments.

a, a^1, a^2, &c., successively hindwards. Those immediately next to them on the right and left at the corresponding levels are referred to as *b*, b^1, b^2, &c., and *c*, c^1, c^2, &c., respectively. The squares still farther away from *b*, b^1, b^2, and *c*, c^1, c^2 are labelled *bb*, b^1b^1, b^2b^2, &c., and *cc*, c^1c^1, c^2c^2, &c., respectively. The square 0·3 mm. anterior to the node is labelled *HMa*; those on the right and left of it *HMb* and *HMc* respectively.

Each square was then removed and grafted between the endoderm and the epiblast of a host embryo of the same age (definitive primitive streak) in a manner described by Waddington (1932).

RESULTS

In the description of the specimens, the letters *HMb*, *HMc*, &c., refer to the level of the donor blastoderm from which the graft was made. The protocol number of the operation is given afterwards. It is not possible to describe here all the important cases. One or two of each level will be described.

HMa region

This region is a square of about 0·3 mm. side in front of the node (*a*) of the primitive streak. It is the region of the presumptive forebrain. Thirty-one grafts from this level were made. Two gave inductions and 11 differentiated into neural tissue.

HMa 30. Length of donor streak 2 mm.; of host streak 1·5 mm. Graft placed in the left anterior region. In the section (Plate 1, fig. 1) an induced secondary neural plate was seen. It was continuous with the host neural tube. The graft seems to have fused with the endoderm of the fore-gut of the host to form a thick neural plate. A considerable amount of mesenchyme is seen scattered round about the secondary neural plate and the graft. The fusion of the graft with the endoderm, especially when the graft lies in the head, has been described by Abercrombie (1937).

HMa 26. Donor streak 2 mm.; host streak 2 mm. In the section (Plate 1, fig. 2) the graft has differentiated into a complete neural tube and seems to have induced a neural plate above it.

HMb and HMc regions

These are the squares of 0·3 mm. side on the right and left sides of the square *HMa* respectively. Ten grafts were made from *HMb* level, of which 4 differentiated into neural plates. Fourteen were made from *HMc* level, 5 of which differentiated into neural plates. In neither level was induction caused. Four grafts developed into hearts.

HMb 1. Donor streak 1·8 mm.; host streak 1·5 mm. Graft placed in the left anterior region. A very beautiful heart, derived from the graft, was seen beating in the specimen (Plate 1, fig. 3) cultured for 24 hours. It seems to be of the same shape and size as that of the host. In section (fig. 4), the graft neural plate is fused with the endoderm of the host.

HMb 6. Donor streak 2 mm.; host streak 1·9 mm. Graft placed in the right anterior region. The graft differentiated into a large neural tube (Plate 1, fig. 5). Even though the neural tube derived from the graft is in contact with the host ectoderm, no induction was caused.

Node region (a)

This is the square of 0·3 mm. side containing the node of the primitive streak. In all 20 grafts were made from this region, of which 17 caused inductions. The capacity for induction seems to be highest at this level of the primitive streak.

a 11. The graft appears to have been incorporated into the host (Plate 1, fig. 6) and has induced a complete secondary axis with neural tube, notochord, and fore-gut. The specimen was fixed after 46 hours of culturing.

b and c regions

These regions are the 0·3 mm. squares immediately to right and left of the node respectively. Thirty grafts of *b* were made, of which 2 caused inductions

and 8 differentiated into neural tissue. Four differentiated only into mesenchyme. Thirty-nine grafts were made from *c*, of which 3 gave inductions; 14 differentiated into neural tissue and 4 into mesenchyme alone.

b 11. Donor streak 2 mm.; host streak 1·4 mm. Graft placed in the left anterior region. In the section (Plate 1, fig. 7) the induced neural tube is seen confluent with that of the host. The graft is differentiated into a neural tube, which is not complete, and there is much mesenchyme. No notochord seems to be associated either with the induced neural tube or the graft.

b 28. Donor streak 1·8 mm.; host streak 1·9 mm. In the section (Plate 1, fig. 8) the graft seems to have fused with the endoderm of the host to form a neural tube, though not a complete one. Associated with the graft is mesenchyme as well as a well-developed heart. On the inner side of the heart there is a tubule lined by endodermal cells. The graft is separated from the host ectoderm by a thick layer of mesoderm, and there is no induction.

c 6. Graft placed in the left anterior region. In section (Plate 1, fig. 9) the graft has differentiated into a neural plate fused with the endoderm, and has induced a neural plate above.

c 28. Donor streak 1·9 mm.; host streak 1·5 mm. Graft placed in the right anterior region. The graft has differentiated (Plate 1, fig. 10) into a neural plate, a notochord, and a group of mesenchyme cells on either side of it, probably forming a somite. The section is an interesting instance showing the interrelationship between the formation of somites and the notochord, which will be considered in the discussion. In this case, although the graft has differentiated into neural tube and notochord, and lies close to the host ectoderm, it has not produced an induction.

c 27. Donor streak 2 mm.; host streak 1·5 mm. Graft placed in the left anterior region. In the section (Plate 2, fig. 11) the graft is seen to have differentiated into a well-developed heart, some mesenchyme and a round mass of cells, probably a somite. At the time of fixation two hearts were seen beating. The heart of the host is larger in lateral extent than that of the graft, and lies opposite to it.

Second level (a^1) of the primitive streak

This is a square of 0·3 mm. side behind the node. In all, 16 grafts were made from this region, 9 of which caused inductions.

b^1 and c^1 regions

These are the 0·3 mm. squares immediately to the right and left of the second anterior level of the primitive streak (a^1). Out of 25 grafts made from the b^1 region, only 1 caused a weak induction, and 5 differentiated into neural tissue. Thirty-three grafts were made from c^1, of which 5 caused inductions and 8 differentiated into neural tissue.

b^1 25. Donor streak 2 mm.; host streak 1·7 mm. Graft placed in the anterior region. The graft seems to have differentiated (Plate 2, fig. 12) into a complete

neural tube; due to the presence of the graft the diverticulum of the fore-gut on that side is withdrawn and is divided into two short diverticula.

c^1 *15.* Donor streak 1·8 mm.; host streak 1·2 mm. Graft placed in the left anterior region. A good induced neural plate is seen in the entire specimen (Plate 2, fig. 13*a*). In section (fig. 13*b*) the induced neural plate is seen widely separated from that of the host. There is no trace of the graft other than a mass of mesoderm.

Third level of the primitive streak (a^2)

This region is a square of about 0·3 mm. sides posterior to the second level of the primitive streak (a^1). Eight grafts were made, of which 3 gave inductions.

b^2 and c^2 regions

These are the 0·3 mm. squares immediately to the right and left of the third level (a^2) of the primitive streak. Out of 16 grafts made from b^2 none caused induction and only 2 differentiated into neural tissue. Thirteen grafts were made from c^2, of which 1 caused induction and 3 differentiated into neural tissue.

c^2 *5.* Donor streak 1·5 mm.; host streak 1·8 mm. Graft placed in the left anterior region. In the entire specimen (Plate 2, fig. 14*a*) as well as in section (fig. 14*b*) an induced neural plate can be seen attached to that of the host in the hind-brain region. No neural tissue has developed in the graft, which is seen in section as a strand of mesoderm beneath the induced neural plate. It seems that the presence of the graft in the heart region has upset the formation and extension of that organ in the host.

c^2 *11.* Donor streak 2 mm.; host streak 1·9 mm. Graft placed in the left anterior region. In the section (Plate 2, fig. 15) the graft is seen to have differentiated into an almost complete neural tube. Round about are a few scattered mesenchymal cells.

Peripheral grafts

The peripheral grafts are difficult to distinguish from the tissues of the host. It seems from the results that none of the peripheral grafts have a capacity for induction and that they differentiate mostly into myoepicardium or mesenchyme.

bb and cc regions

These grafts were taken 0·4–0·6 mm. laterally from the node of the primitive streak on right and left respectively. Out of 8 grafts of *bb*, 1 can be recognized as mesenchyme. Fourteen grafts of *cc* were made, of which 3 were recognized as forming either mesenchyme or myoepicardium. The remaining grafts of *bb* and *cc* could not be identified.

bb 3. Donor streak 2 mm.; host, early head process stage. Graft placed in the right anterior region. In the section (Plate 2, fig. 16) the graft differentiated into a mass of mesenchyme arranged round roughly circular cavities.

b^1b^1 and c^1c^1 regions

These regions are 0·4–0·6 mm. to the right and left sides of the second level of the primitive streak. Seven grafts of b^1b^1 were made, of which 2 differentiated into myoepicardium. Of 9 grafts from c^1c^1, 2 differentiated into myoepicardium and mesenchyme.

b^1b^1 1. Donor streak 2 mm.; host streak 1·7 mm. Graft placed in the left anterior region. In section (Plate 2, fig. 17) the graft has differentiated into a round thin-walled structure surrounded by a thin layer of mesenchyme. It was beating at the time of fixation; thus it may perhaps be myoepicardium. The surrounding layer looks like somatic mesoderm, but its nature cannot be definitely ascertained.

c^1c^1 4. Donor streak 2 mm.; host streak 1·9 mm. Graft placed in the left anterior region. The graft (Plate 2, fig. 18) seemed to have differentiated into a well-developed heart (endocardium and myoepicardium) and a group of mesenchyme cells. Beneath the mesenchyme is seen a small, yet complete neural tube. It has probably been formed by regulation. The heart derived from the graft is almost as big as the heart of the host.

b^2b^2 and c^2c^2 regions

These levels are 0·4–0·6 mm. to the right and left respectively of the third level of the streak (a^2). Six grafts of b^2b^2 were made, of which 2 differentiated into recognizable mesenchyme or myoepicardium. Of 11 grafts of c^2c^2, 3 developed into myoepicardium or mesenchyme.

DISCUSSION

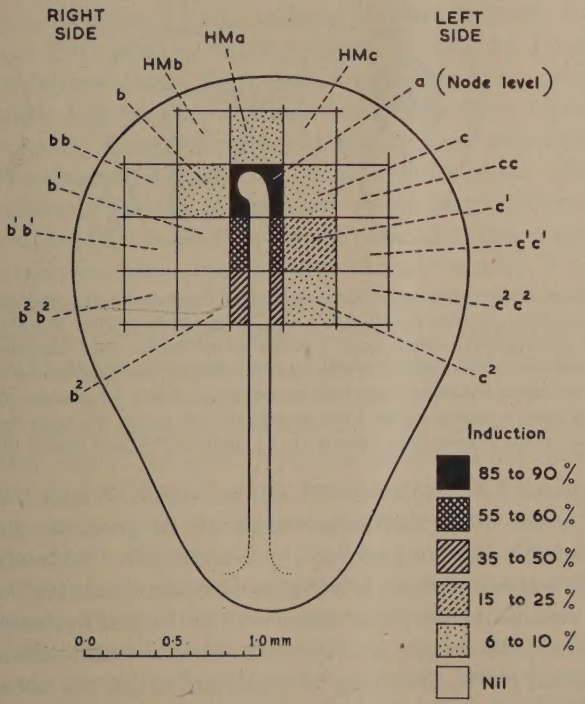
From the results of the present work it appears that besides the anterior third of the primitive streak, the regions at least 0·3 mm. in front and to the side of it have an inducing capacity, though to a much lesser extent. This does not seem unlikely from consideration of the results obtained by Abercrombie (1950), who found that after reversal of a variety of different parts of the streak many cases of regulative development were obtained, showing that the primitive streak is subject to control by the surrounding blastoderm.

Bautzmann (1926) also found that in amphibia the organizing capacity is not restricted to the blastopore alone, but is spread over a quadrant of the egg.

On the basis of the results obtained in the present work, a map showing the extent of the organization centre in the chick blastoderm at the definitive streak stage may be presented. Text-figs. 2 and 3 show that the frequency of induction is highest at the node of the primitive streak, and falls off laterally as well as along the axis. It seems that there are antero-posterior and medio-lateral gradients of inducing activity. From the present work nothing can be said about the nature of the gradients, but it is clear that the regions (b, c, c^1) near the notochord have a greater inducing capacity than the parts (bb, b^1b^1, cc, c^1c^1, &c.) away from it.

	0/10 0%	2/31 6%	0/14 0%	
0/8 0%	2/30 7%	17/20 85%	3/39 8%	0/14 0%
0/7 0%	0/25 0%	9/16 56%	5/33 15%	0/9 0%
0/6 0%	0/16 0%	3/8 38%	1/13 8%	0/11 0%
0/21	2/81		9/99	0/34

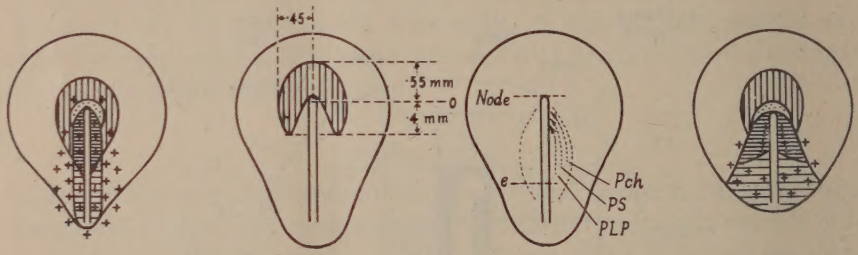
TEXT-FIG. 2. A map showing extent of the organization centre in a blastoderm of a definitive streak stage. The two figures at the top of each square give, first, the number of inductions obtained when that region was used as a graft, and, second, the total number of such grafts made. In the lower part of the square, the number of inductions is expressed as a percentage of the number of grafts.



TEXT-FIG. 3. Graphic representation of the extent of the organization centre.

It is interesting to note that the frequency of induction as well as of differentiation, in the grafts from the right (b^1, b^2, &c.), is less than that at the corresponding levels from the left (c^1, c^2, &c.). The grafts from the right at these levels have not caused any induction. It is probable that the asymmetry of structures shown in the adult bird is seen even at such an early stage of development. Reference to the asymmetry of the node at the primitive streak stage has been made by Jacobson (1938). The superior developmental capacity of the grafts from the left over those from the right, at the stage of the head process blastoderm, has been shown by Rawles (1936, 1943) and Rudnick (1932), with the chorio-allantoic technique.

A correlation between induction and the self-differentiation of the graft into neural tissues was suggested by Waddington (1952). The present results agree in some respects with this suggestion. The more peripheral grafts, from *bb*, b^1b^1, b^2b^2, and *cc*, c^1c^1, c^2c^2, &c. (Plate 2, figs. 16, 17, &c.) which have never differentiated into neural tissue have not caused any induction, while some grafts from *b*, *c*, c^1, &c., close to the anterior levels of the primitive streak, have differentiated into neural tissue, and have induced (Plate 1, figs. 7, 9, &c.). At the same time it must be remembered that some grafts (Plate 1, figs. 5, 10, &c.) which have differentiated well have not produced inductions.



TEXT-FIG. 4. Maps of the presumptive areas in a chick blastoderm of a definitive streak stage. A, after Pasteels (1937); B, after Spratt (1947 *a*, *b*, 1952, 1955); C, after Waddington (1952). In Figs. A and C the presumptive neural tissue is indicated by vertical lines, the presumptive chorda by dots, the presumptive axial mesoderm by close horizontal lines, and non-axial mesoderm by spaced horizontal lines; already invaginated mesoderm is shown by crosses. In Fig. B the presumptive neural plate is shown by the lined area in the left figure, while on the right there are indications of the presumptive chorda (*Pch.*), somite (*PS*), and lateral plate (*PLP*).

It was suggested by Hoadley (1927), Gräper (1929), Wetzel (1929), Umanski (1931), and Dalton (1935) that induction should be primarily due to the axial mesoderm; and this view was adopted by Waddington (1932) when he showed that the streak is able to induce. It is thus worthwhile discussing the position and extent of the various presumptive tissues with the help of the results obtained in the present work. But in any isolation experiments (chorio-allantoic, *in vitro*, intrablastodermal grafts) there may be regulation within the isolate, and organs may appear from regions which are not their presumptive origin. With this

caution in mind, an attempt will be made to discuss the position and extent of the various presumptive tissues.

The localization of the presumptive tissues in the early and definite streak stages has been mapped out by several investigators, such as Gräper (1929), Wetzel (1929), Pasteels (1937), Waddington (1932, 1952), Rudnick (1938 *a*, *b*, *c*), Spratt (1942; 1947 *a*, *b*; 1952; 1955), and several others. It is beyond the scope of the present work to discuss all the maps. The maps given by Pasteels (1937), Spratt (1947 *a*, *b*; 1952; 1955), and by Waddington (1952) are shown in Text-fig. 4.

Presumptive neural tissue

In the definitive streak stage, according to Pasteels, the presumptive neural tissue is crescentic in shape, the two arms tapering off and extending towards the middle of the streak. In Spratt's map the neural plate is 'oval' in outline (the node lying in the centre), extends about 0·25 mm. anterior, 0·4 mm. posterior, and 0·45 mm. lateral to the primitive pit (Spratt, 1947*b*, 1952). In Waddington's maps the presumptive neural area is in the shape of a horseshoe, the two arms extending along about one-third of the streak. No measurements are given either by Pasteels or by Waddington.

In the present work neural differentiation was obtained from the regions *HMa* (Plate 1, figs. 1, 2) (up to 0·3 mm. in front of the node level); *HMb*, *HMc* (figs. 5, 6) (lateral to *HMa*), and up to 0·6–0·9 mm. (Plate 2, fig. 15) behind the node level of the primitive streak. The posterior extension of the neural tissue in the definitive streak stage has been studied by several workers. Wetzel (1924) at first failed to get neural tissue from the posterior regions behind the node, though later (1926) he obtained it from some of the posterior pieces. Hunt (1932), using the chorio-allantoic technique, could not obtain neural differentiation behind the node level, but Dalton (1935) using the same technique succeeded in getting neural differentiation from about 0·5 mm. behind the node. Waddington (1935) obtained neural tissue *in vitro* from parts of a blastoderm as far back as 0·7 mm. posterior to the pit. On the other hand, Rudnick (1938*b*) and Spratt (1952), using the *in vitro* technique, claimed that neural plate never developed in posterior pieces cut from about 0·4 mm. behind the pit. As mentioned above, the region 0·6–0·9 mm. behind the node has formed perfect neural tube (Plate 2, fig. 15), and, moreover, one of the grafts from the same level has caused induction as well (fig. 14 *a*, *b*). Waddington (1952) has suggested that the boundary between the inducing and non-inducing region is shown by the limit at which the neural tissue can develop in isolates *in vitro*; however, Abercrombie & Bellairs (1954) and Islam (1953) have reported inductions through the posterior third of the primitive streak, which is the site of presumptive lateral plate mesoderm, and which itself has not so far been described as forming neural tissue.

The capacity for self-differentiation into neural tissue independently of the mesoderm has been shown only for the fore-brain, by Waddington (1932),

Rudnick (1938*b*), and Spratt (1942, 1947*c*). In the present work the graft *HMa 28* (not shown), which was labelled with radioactive methionine, clearly shows the self-differentiation of the graft (presumptive fore-brain) independently of the mesoderm. However, no other part of the central nervous system besides the fore-brain is described so far as possessing this capacity, and thus the differentiation of neural tissue from the posterior levels may be due to its induction by the underlying mesoderm as suggested by Waddington. In those cases in which neural tissue is produced by fragments from the posterior end of the streak, the mesoderm has probably regulated to a more 'anterior' condition before inducing the neural tissue.

Presumptive axial mesoderm (somites and notochord)

In the definitive streak stage the presumptive axial mesoderm in Pasteels's map is seen as a narrow band occupying more than half the length of the streak. This corresponds more or less with the position and extent of that tissue shown by Gräper, Wetzel, and Waddington. Spratt (1955) makes a further distinction between the somite centre, restricted to about 0·1–0·2 mm. posterior to the primitive pit, and the prospective somite cells found as far as 0·6–0·8 mm. behind the node.

In the present study it is observed that the differentiation of somites and notochord, the typical structures of the axial mesoderm, is rarely seen except in a few grafts from region *c* (Plate 1, fig. 10; Plate 2, fig. 11). The mesenchyme in these grafts tends to arrange itself into round masses appearing like somites.

The tendency of the mesoderm to form somites in some grafts from *c* may be due to the fact that these grafts lie adjacent to the node level of the primitive streak, which is perhaps the region of the notochord. Interrelation between the chorda and somites has been suggested by Spratt (1942) and Islam (1953) in the chick, and by Yamada (1940) and others in Amphibia, although Abercrombie & Bellairs (1954) have doubts about the situation in the chick.

The lateral extent of the axial mesoderm seems to correspond to a narrow band, as shown in Pasteels's mapping scheme. The more peripheral grafts, *bb*, b^1b^1, b^2b^2, *cc*, c^1c^1, &c., in the present study have shown poor differentiation, except for myoepicardium.

In the present study the notochord is not seen to differentiate from any region other than the node, except in one single instance (Plate 1, fig. 10), in which it is formed from the region adjacent to the node level on the left (*c*). It is difficult to say whether in this case the notochord is formed by regulation, or if its presumptive area is spread out laterally as far as this.

It is also seen that the inductions, when caused by lateral grafts, were mostly neural inductions with little or no accompanying mesenchyme. The induced neural plate was not associated with notochord in any case (Plate 1, fig. 9; Plate 2, figs. 13*b*, 14*b*). It may be that the grafts from these various lateral levels did not contain the region of the presumptive notochord and that the absence of

notochord in the induction is to be accounted for by this fact. On the other hand, one might ask, are the notochordal inductions specific? Or, in other words, are there different substances for neural and mesodermal inductions?

Heart-forming areas

In the definitive streak stage Hunt (1932) found the highest frequency of heart formation in the pieces lying about 0·3 mm. behind the primitive pit. Rudnick (1938*b*), with *in vitro* culturing technique, reported heart differentiation as far back as 1 mm. behind the pit. In the head process stage Rawles (1943) obtained the formation of heart anteriorly up to 0·5 mm. and posteriorly 0·4 mm. from the primitive pit. The lateral extent in that stage was found to be from 0·2 mm. to each side of the pit, to a region at least 0·4 mm. distant.

In the present study differentiation of heart has occurred in the grafts from various levels. To give a few instances, the graft *HMb* (0·15–0·45 mm. lateral to the axis) has formed a beautifully developed heart beating at the time of fixation (Plate 1, fig. 3). Hearts were seen to differentiate from the grafts from regions *b* and *c* (Plate 1, fig. 8; Plate 2, fig. 11, &c.) and from a number of peripheral grafts from b^1b^1, c^1c^1, c^2c^2 (Plate 2, figs. 17, 18, &c.). The peripheral grafts c^1c^1, c^2c^2, in which the heart-muscle seems to develop, are 0·45–0·75 mm. lateral to the various levels of the primitive streak, and some c^2c^2 (not shown), were a similar distance behind the node. It appears from this that in the primitive streak stage the heart-forming area is larger than in the head process stage as described by Rawles (1943); this reminds one of the gradual contraction of the area found by Ebert (1953) to contain cardiac myosin.

SUMMARY

1. Blastoderms of the definitive primitive streak stage were cut into as many as eighteen squares of about 0·3 mm. side (cf. Text-fig. 1). The inducing capacity of each small piece was tested by transplanting it into the area pellucida of a host blastoderm (in the definitive streak stage), cultured by an *in vitro* technique. In all, 303 grafts were made and 270 histologically examined.

2. From the results obtained it seemed probable that the grafts from the level immediately (0·15–0·45 mm.) in front of the node, and a similar distance lateral to the node and the anterior third of the primitive streak, have an inducing capacity, though to a much lesser extent than the node itself.

3. The results of the present work make it probable that the inducing capacity falls off not only along the axis of the blastoderm but also medio-laterally. It appeared as if there were gradients in inducing capacity along the longitudinal and transverse axes of the blastoderm. A map showing the extent of the organizer in the blastoderm of a definitive streak stage is given (Text-figs. 2 and 3).

4. The frequency of induction and differentiation was higher in the grafts

from the left side of the blastoderm than in those from the right. This is perhaps the earliest manifestation of the asymmetry of structures found in the adult bird.

5. Peripheral grafts appeared to differentiate only into myoepicardium or mesenchyme.

6. The map of presumptive areas in the definitive streak stage chick blastoderm is discussed with reference to the results obtained.

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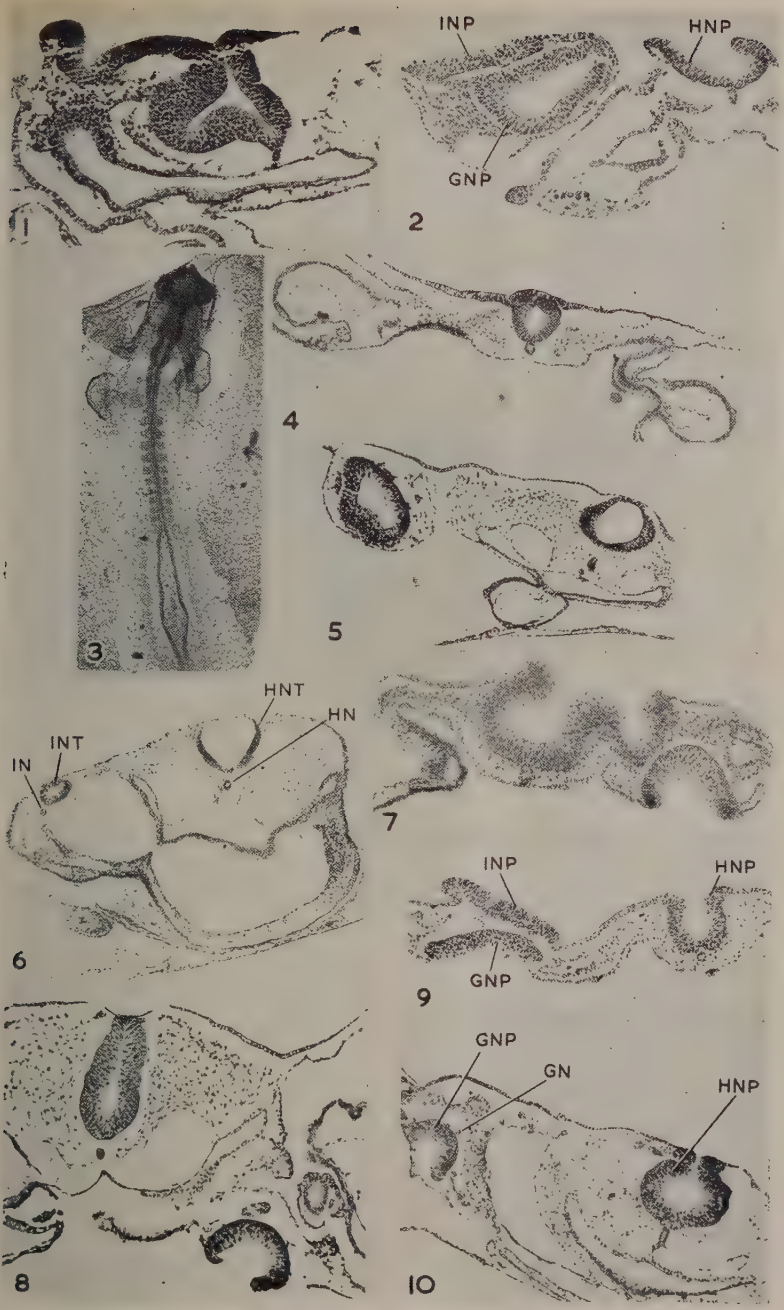

L. MULHERKAR

Plate 1

FIG. 14*a*. c^2 *5*. Whole mount (×10).
FIG. 14*b*. c^2 *5*. Section of the same through the heart region (×70, sides reversed).
FIG. 15. c^2 *11*. Section (×70).
FIG. 16. *bb 3*. Section through the graft (×320).
FIG. 17. b^1b^1 *1*. Section (×80).
FIG. 18. c^1c^1 *4*. Section (×80).

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A Physiological Interpretation of the Mechanism Involved in the Determination of Bilateral Symmetry in Amphibian embryos

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INTRODUCTION

VERY great interest has been taken in the question of the nature of those factors which determine the bilateral symmetry in amphibian embryos. Numerous experimental observations have been published, and various hypotheses and theories have been advanced to account for the results. The problem has been thoroughly discussed in several reviews (of which many of the more recent will be quoted below) and it would for this reason alone be out of place to review the literature in the present context. For orientation, the present views upon the problem will be presented in brief outline.

MORPHOLOGY OF THE DETERMINATION PROCESS

It seems that factors responsible for bilateral symmetry are located partly in the fluid cytoplasm in the interior of the egg, and partly in the more solid cortex. It has previously been thought that the organization of the cytoplasm was determined by the influence of gravity, the constituents being distributed simply according to a density gradient. This idea has had to be abandoned, however, since it has been shown that the cytoplasmic inclusions (especially the yolk granules) are arranged in a definite pattern (Ancel & Vintemberger, 1948; Pasteels, 1951; see also Lehmann, 1945). This organization is labile to some extent, and may be changed experimentally. The influence of gravity on this distribution may be shown by inversion of the egg. Thus, when the egg is kept upside down, the large heavy yolk granules normally occupying the vegetal hemisphere, and the lighter cytoplasm in the animal hemisphere, will partly change places. The cortex or pellicle of the egg comprises a layer of pigmented cytoplasm, a very thin hyaline layer, and the cell-membrane proper (Ancel & Vintemberger, 1948; Dalcq & Dollander, 1948; Holtfreter, 1948). There is yet

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another structure which may be considered to belong to the cortex, viz. the coat (Holtfreter, 1943; Ancel & Vintemberger, 1948; Dollander, 1951). This coat is a thin extracellular pigmented protein layer covering the entire egg surface.

Among the cytoplasmic factors the distribution of yolk is of importance; the invagination generally seems to occur near the margin between a yolk-covered and a yolk-free area (cf. Weigmann, 1926, 1927; Ancel & Vintemberger, 1948). Anomalous yolk distribution obtained by inversion may thus lead to abnormal invagination. It has been suggested by Holtfreter & Hamburger (1955) that 'yolk plays a passive role in this respect, serving merely as a support to direct cytoplasmic shifting'. The material in the yolk-free region may also be of importance. Rather little is known about the composition of the cytoplasm of amphibian eggs. Using cytochemical methods Brachet has shown that both RNA and proteins containing reactive sulphydryl groups were located almost exclusively in the animal hemisphere, maybe partly associated with mitochondria-like inclusions (cf. Brachet, 1945). The possible morphogenetic influence of this part of the egg's cytoplasm has been stressed by some authors (see the discussion by Lehmann, 1945).

The ideas about the relative influence of the cortex vary widely. Lehmann (1945) seems to maintain the view that the cortex has next to no influence, and certainly the fact that invagination under experimental conditions may occur almost anywhere on the egg surface, may seem to support this view. The other extreme is represented by the theory of Dalcq & Pasteels (1937, 1938), which postulates a cortical gradient field of specific morphogenetic substances with centre in the grey crescent.

The truth may lie somewhere between these extreme views. It cannot be questioned, however, that the formation of the grey crescent in the egg cortex is an event of great importance for the determination of the dorso-ventral axis. The respective functions of the cytoplasmic and the cortical factors have been shown in inversion experiments with eggs after the appearance of the grey crescent. Under these conditions the invagination is found to occur at the margin of yolk closest to the original grey crescent (Weigmann, 1926, 1927; Penners & Schleip, 1928 *a*, *b*; Pasteels, 1938, 1939; see also the discussion by Ancel & Vintemberger, 1948). This observation shows that the cortical factors, localized in the grey crescent, are stabilized before the cytoplasmic factors. It is thus possible to distinguish between the function of the two sets of factors. The cortical 'dorsalization' factors seem to be responsible for determining in which region of the egg invagination will occur. The cytoplasmic 'symmetrization' factors determine, on the other hand, the direction of invagination and thus the plane of bilateral symmetry.

From the literature quoted above it appears that the cytoplasmic and cortical factors taken together establish the axial polarity, and thus the bilateral symmetry. The localization of the grey crescent determines the future dorsal side. The next question obviously concerns which factors determine the site of the

grey crescent. This problem has been thoroughly discussed by Ancel & Vintemberger (1948), who themselves have carried out a series of extremely careful studies on the mechanism involved in the determination of the localization of the grey crescent. It is necessary here to deal with this question in some detail, because the nature of this mechanism is of importance for the physiology of the determination process. The following description of the processes going on in the egg up to the end of grey crescent formation is, when not otherwise stated, based upon the work of Ancel & Vintemberger. It should be mentioned that most of this work has been done on eggs of *Rana fusca*. For reasons mentioned below the interpretation given here deviates on several points from that given by Ancel & Vintemberger, to whose work the reader may refer for further details.

A few minutes after activation or fertilization, a contraction of the cortex occurs, having its centre at the animal pole. As a consequence of this contraction the edge of the pigmented cortex is pulled away from the visible yolk margin, and a grey ring or belt is formed below the egg equator. On sections this process is revealed by thickening of the cortical pigment layer and wrinkles on the surface round the animal pole. It seems reasonable to believe that this contraction occurs in the very elastic surface coat (cf. Holtfreter, 1943). The contraction is reversible; after about 10 minutes the pigment border has more or less returned to the original location. If the egg is in water so that the normal swelling of the jelly may occur, the egg will begin the rotation of orientation as soon as the cortical contraction is over. During this rotation the egg orientates itself, under the influence of gravity, so that the animal-vegetal axis is vertical. The whole egg rotates inside the vitelline membrane, and it may be that the cortical contraction prepares the rotation by disrupting the immediate contact between vitelline membrane and egg surface. It should be mentioned in this context that according to Holtfreter (1943) there seems to be a more firm contact between vitelline membrane and egg surface at the animal than at the vegetal end of the egg. The rotation takes about 20 minutes, and subsequently the perivitelline space begins to form. It has been clearly shown by Ancel & Vintemberger that the primary mechanism involved in this process is the osmotic absorption of water from the outside. A precondition may be the already mentioned disconnexion of the contact with the vitelline membrane at the animal pole. In the shaping of the perivitelline space other factors may be involved, e.g. gravity and the elastic tension of the coat (cf. the observation that calcium influences the size of this space). The increase in size of the perivitelline space goes on for about 90 minutes.

The next stage, the process leading to the formation of the grey crescent, goes on between the first and second hour after fertilization, and is called by Ancel & Vintemberger the rotation of symmetrization. According to these authors the entire cortex rotates about 30° around an axis perpendicular to the plane of symmetry. In this way the pigmented cortical layer is lifted at the dorsal side,

thus forming the grey crescent. At the ventral side only the outermost layers of the cortex cross the yolk margin, as indicated by a slight accumulation of pigment at the ventral side of the vegetal hemisphere. The resistance of the yolk against the cortical rotation is thought to explain why only this small fraction of the cortex can cross the border. Ancel & Vintemberger can only suggest gravity as the force driving this rotation of the cortex while the interior of the egg remains static. It is hardly possible, however, to imagine that any great differences could arise between the accumulation of material at the dorsal and ventral sides. Especially when the above-mentioned resistance at the vegetal border is considered, it seems necessary to conclude that such rotation is not possible. There are several experimental observations which do not conform with this rotation, but before we discuss them, the experiments of Ancel & Vintemberger must be described in further detail. In their studies these authors used three different methods for determining the plane of bilateral symmetry: forced inclination (135°) of the animal-vegetal axis (in a humid chamber), directed rotation of orientation, and fertilization. In the first method the middle of the grey crescent will be at that point of the vegetal border which was highest in the inclined egg. In the second method the plane of symmetry is determined by the plane of rotation, again the highest point on the border will be in the middle of the grey crescent. Finally, in the third method the point of sperm entrance determines the plane of symmetry, and the grey crescent will form on the side opposite that of the sperm entrance. The common feature in the effects of these three procedures seems to be that at the dorsal side the contact with the yolk is disrupted in a narrow strip of the very thin vegetal cortex. The mechanism involved in the two first methods is gravity: yolk sliding down when the egg is kept inverted; and when the egg is rotated yolk moves faster than cortex, thus coming to lie slightly ahead of the latter. In the fertilized egg an asymmetrical contraction seems to occur, with centre at the point of sperm entrance. On sections this contraction is indicated by the accumulation of cortical pigment around the point of sperm entrance; a similar phenomenon is not observed in activated eggs. Whereas the effect of this contraction is very slight on the ventral side, due to the short distance to the pigment border, the contraction is quite considerable at the dorsal side, so that a distinct asymmetry is observable in the pigment distribution. For the following discussion it is necessary to make one assumption, viz. that the contact between yolk and cortex is so firm that only slight movements of the vegetal cortex are possible. If we imagine that a strip of this thin cortex is deprived of the yolk lining, we must expect that the tension in the cortex of the animal part of the egg is no longer in equilibrium. The elastic forces of the cortex (coat) will therefore slowly and gradually stretch this strip until a new equilibrium is reached. The area comprising this region of stretched material would be the grey crescent. It is reasonable to assume that the whole animal cortex slides ventrally, as indicated by experiments with electrolytic marking made by Ancel & Vintemberger, and likewise, as mentioned above,

that the deeper lying cortical material is accumulated at the ventral side. The excess coat seems to be incorporated or absorbed by the immobile coat at the ventral side of the vegetal end, as indicated by pigment accumulation. Such a coat reabsorption process has been described by Holtfreter (1943). The explanation given here is in agreement with the results of Ancel & Vintemberger except for the fact that their marking experiments also indicated movement in the direction from the vegetal pole towards the lower edge of the grey crescent. This observation may find an explanation in the fact that a coagulum fixed at the vitelline membrane may make a trace on the surface when the egg is moving inside the membrane. If the vitelline membrane egg-surface connexion is firmer at the animal side, it may be that the vitelline membrane with the fixed coagulum has been moved along the vegetal surface during the grey crescent formation, thus making the observed trace. There are some observations made by Ancel & Vintemberger which conform better with the mechanism suggested here than with the proposed rotation. Thus, the marking experiments show that dorsolateral points do not move in planes parallel with the plane of symmetry, but unexpectedly they converge towards this plane. Also, the vital staining experiments of Banki (1929) show that a stretching occurs at the dorsal, but not at the ventral side (cf. Banki's pictures with those of Holtfreter (1943) showing stretching of the coat during wound-healing).

It should be mentioned that in eggs activated with the animal pole upwards, no determination of the grey crescent site takes place; it may appear at any side of the egg. No asymmetry is to be seen in sections. According to the mechanism outlined above, a very slight asymmetry would suffice to establish that distortion of the equilibrium in the cortical tension needed for the formation of the grey crescent. Such asymmetry might arise during the first cortical contraction.

In perfect agreement with the ideas outlined above are the observations on the permeability to Nile blue of the amphibian egg (Dalcq & Dollander, 1948; Dollander & Melnotte, 1952). It was found that before fertilization the dye penetrates to the same extent at all points of the surface; after fertilization and symmetrization the permeability is reduced at the animal and ventral sides, increased at the dorsal and vegetal regions. This result may be the combined effects of the symmetrical and the asymmetrical contraction. Unfortunately no experiments were made on fertilized eggs before formation of the grey crescent. It would be interesting to see if in these eggs the permeability of the vegetal region was increased, in which case it would be necessary to ascribe this phenomenon to the first symmetrical cortical contraction.

As a conclusion to this discussion on the mechanism involved in the determination of the grey crescent location it may be stated that the process called by Ancel & Vintemberger the rotation of symmetrization in all probability is a stretching or a contraction of symmetrization.

This summary presents the current ideas, in so far as they have been developed upon the basis of morphological observations. The problem has also been

attacked from the physiological point of view, and this work will be discussed in the following section.

PHYSIOLOGY OF THE DETERMINATION PROCESS

Before we begin to discuss the physiological aspects of the mechanism involved in the determination of the bilateral symmetry, we may briefly consider the problem of axial determination in biological systems. It seems necessary to assume that the organization of the unfertilized egg is radially symmetrical. This is indicated partly by the rotation and fertilization experiments mentioned above, and partly by the existence of the so-called exogastrulation, an abnormal pattern of gastrulation which has been observed to occur spontaneously, but also may be provoked experimentally (see Holtfreter, 1933). The mechanism involved in exogastrulation seems to be that invagination, instead of beginning only at one side of the egg, occurs along the entire equatorial zone simultaneously. This phenomenon tends to emphasize that the basis of the dorsoventral polarity which is necessary for a normal gastrulation is the establishment of asymmetric conditions in the egg. This asymmetry at first only involves a quantitative difference; even in normal development invagination occurs at all sides of the egg, but the process is delayed at the ventral side. The possibility that axial determination has its origin in quantitative differences has particularly been emphasized by Child (1941, 1946). Child has also stated the view that external factors must be involved in the establishment of axial polarity. When the idea of Child is applied to the present problem the importance of the formation of the grey crescent is that it allows an axial gradient to be established under the influence of external factors. Child postulates furthermore that the gradients involved in axial determination are metabolic ones. It will be seen that the hypothesis advanced here is in agreement with Child's theory.

It will facilitate the following discussion if we begin by presenting the hypothetical interpretation, but before this is done we may briefly state the following demands to be made of this hypothesis: (1) The interpretation must, as far as possible, account for all morphological, physiological, and biochemical results obtained in studies of this phase of embryonic development. (2) It must be epigenetic rather than preformationist, i.e. the assumptions as to the organization of the egg should be as few as possible. (3) It should be formulated in such terms that experimental verification is possible.

It will now be attempted to show that in the literature strong support may be found for the following postulate: The determination of the dorsal area consists in creating such conditions that the rate of oxygen consumption can be much higher in this area than in any other part of the egg. On this basis we will discuss the possible nature of the cytoplasmic and cortical factors which are involved.

It is well known that the normal segmentation process up to the beginning of gastrulation may occur anaerobically, indicating that the energy requirements

for this process are quite low. This is corroborated by the observations that consumption of energy reserves (carbohydrate) is low (Brachet & Needham, 1935; Gregg, 1948), that no RNA or protein synthesis is demonstrable (Brachet, 1945; Løvtrup, 1955), and that the DNA synthesis which is associated with segmentation goes on at the cost of preformed reserves (Zeuthen, 1951; Hoff-Jørgensen & Zeuthen, 1952; Hoff-Jørgensen, 1954; Løvtrup, 1955; Gregg & Løvtrup, 1955).

In contrast to this, gastrulation cannot occur under anaerobic conditions (see Brachet, 1945). In the normal amphibian embryo the respiratory rate shows a slight increase during early development (segmentation), and this increase continues up to the end of gastrulation, following an exponential curve (Atlas, 1938). This respiratory increase must primarily represent the energy consumption of all those processes involved in the normal gastrulation process (cell-divisions and cell-movements, synthesis of various chemical compounds, &c.). This is also suggested by the observation that in lethal hybrids, in which development is blocked before gastrulation, hardly any respiratory increase is found (Barth, 1946; Chen, 1953; but cf. Brachet (1954) who observed respiratory increases in some hybrid species). Likewise the glycogen consumption is very low in hybrids compared with normal embryos (Gregg, 1948). We may thus conclude that among the processes involved in gastrulation some are dependent upon the simultaneous occurrence of oxidative reactions. The main task of these reactions is presumably to supply energy. We must infer from this that the dorsal region, in which invagination occurs, and in which high metabolic activity can be demonstrated (cf. below), has a higher rate of oxygen consumption than other parts of the embryo.

For the supply of energy by oxidation it is necessary to have an energy source, mitochondria, and oxygen. We may consider first the question of the oxygen supply. It will be realized that this may be influenced by the cortex, in so far as the passage of oxygen into the egg may be controlled by a membrane of low permeability. The protein coat which surrounds the mature amphibian egg has extremely low permeability to water (Krogh, Schmidt-Nielsen, & Zeuthen, 1938; Holtfreter, 1943; Prescott & Zeuthen, 1953). There is no reason to believe that a membrane having a low permeability to water is not also relatively impermeable to oxygen (cf. Krogh, 1937), and it therefore seems rather certain that the supply of oxygen is limited by the coat. The stretching of the protein coat which in the previous section was shown to be associated with the formation of the grey crescent leads to increased permeability to dyes (Dalcq & Dollander, 1948; Dollander & Melnotte, 1952). We may expect that the permeability is increased to oxygen also, which is the condition necessary for establishing the postulated axial metabolic gradient. In this way those energy-requiring processes may be initiated which eventually lead to gastrulation. We do not know much about the chemical nature of these processes, except that they include synthesis of DNA, RNA, and proteins (see e.g. Hoff-Jørgensen & Zeuthen, 1952;

Brachet, 1945; Løvtrup, 1955). The energy source and the mitochondria must be localized in the cytoplasm. As far as the former is concerned, we know that the principal energy source during early development is glycogen (see e.g. Brachet & Needham, 1935; Gregg, 1948; Løvtrup, 1953*a*; Løvtrup & Werdinius, 1957). This substance is mainly confined to the animal hemisphere in the early amphibian gastrula (Gregg & Løvtrup, 1950). During gastrulation considerable amounts of glycogen disappear in the dorsal region (Heatley & Lindahl, 1937; Jaeger, 1945). It may be argued that glycogen may disappear without being oxidized; it might equally well be used for synthetic purposes. This argument does not hold, however, for except at high temperatures the oxygen consumption corresponds closely to the carbohydrate utilization (see Løvtrup, 1953 *a*, *b*; Løvtrup & Werdinius, 1957). Mitochondria are present in the amphibian egg (Boell & Weber, 1955), but nothing is known about their distribution.

Under normal conditions the grey crescent forms at the yolk margin, and invagination also occurs in this region. The present hypothesis assumes that the distribution of glycogen and mitochondria is radially symmetrical, but otherwise it only demands that they should be present at the yolk margin. Whether they are localized in a ring (marginal plasm, see Lehmann, 1945), in a layer on top of the yolk or are evenly distributed in the animal hemisphere is of no consequence for the hypothesis. It was mentioned in the preceding section that the cytoplasmic factors may determine the direction of invagination. It is very difficult to imagine that factors freely dispersed in the cytoplasm can exert such influence. If, therefore, glycogen and mitochondria are not fixed to certain structures, the question may be raised whether it is simply the direction of the yolk margin which determines the direction of invagination. Even if passively, the yolk would thus play an important role. It is not possible at the moment to decide this question, but it should be emphasized that there may be two types of cytoplasmic factors, viz. glycogen and mitochondria, which are involved in the establishment of the metabolic gradient, and yolk, which may determine the direction of invagination.

It is important to test the consequences of the hypothesis outlined above. The main point is that in the intact egg the oxygen supply limits the rate of oxygen uptake. This is supported by the fact that the oxygen consumption in homogenates is considerably higher than in whole eggs (Brachet, 1934; Spiegelman & Steinbach, 1945). Likewise, Needham (1942) has calculated from results on the oxygen uptake of explants that they respire at a rate which is about three times higher than in the intact egg.

It is possible to interpret these results differently. Thus the homogenate experiments were thought to represent a change in the substrate-enzyme orientation, and the explant results were considered a result of the inertness of yolk. The results obtained by comparing the oxygen uptake in dorsal and ventral explants may speak more convincingly in favour of the present hypothesis. As mentioned above, it has been possible from the disappearance of glycogen to infer that the

metabolic rate is much higher in the dorsal than in the ventral side, but it has never been possible to show very striking differences between the respiration of ventral and dorsal explants (see, for example, the detailed discussions by Needham, 1942, and Brachet, 1945, and the recent papers by Ornstein & Gregg, 1952, and Sze, 1953). This lack of agreement between expected and observed results may find a natural explanation if, as suggested here, the difference in metabolic rate is caused not by differences in the content of cytoplasmic factors involved in respiration, but by the rate of oxygen supply. In the explants, oxygen may diffuse through the exposed surfaces of the cells inside the coat, and the differences in oxygen uptake between the explants may therefore be quite low. The possibility exists that a coat may be formed at the exposed surfaces (see Holtfreter, 1943). If this happens, the oxygen consumption should be reduced for both explants, but it is not possible to foretell the consequences with respect to the difference in respiration between the explants.

With oxygen diffusion as a limiting factor, the results obtained on consumption of energy sources at different temperatures (Løvtrup, 1953*b*) may find a natural explanation. It was observed that during early development there was a discrepancy between loss of energy reserves (carbohydrate) and oxygen consumption, which led to the conclusion that considerable quantities of carbohydrate were lost without being oxidized. As the increase of diffusion rate with temperature is much lower than that of chemical reactions, the inefficient carbohydrate utilization may reflect that the oxidation of lactic acid formed by glycolysis is lagging behind due to lack of oxygen. In this connexion it should be mentioned that measurable amounts of lactic acid are formed in the embryos under aerobic conditions, but a considerable increase is observed during anaerobiosis (Lennerstrand, 1933; Cohen, 1955). The very low temperature coefficient observed for respiration during the early developmental stages also corroborates the explanation given above (Løvtrup, 1953*b*).

The strongest support in favour of the suggestion that the supply of oxygen is a limiting factor may perhaps be found in the results of Cohen (1955) showing that the amphibian embryo, up to the end of gastrulation, is partially anaerobic. This was indicated by a burst of oxygen uptake which follows transfer of embryos from nitrogen or air to an oxygen atmosphere. Cohen has discussed the possibility that the oxygen supply into the interior of the egg may be limited by the rate of diffusion. Some calculations which he carried out to test this point did not give an unambiguous answer. As Cohen did not take into consideration the possible existence of a membrane acting as a diffusion barrier, the results of his calculations do not invalidate the present hypothesis. They are important, however, because they indicate that the rate of diffusion of oxygen in the cytoplasm is slow enough to permit a gradient of oxygen supply when the permeation is limited to a certain region of the egg surface.

It was observed by Brachet (1934) that the oxygen consumption is almost independent of the oxygen tension. This finding is neither compatible with

Cohen's results nor with the ideas advanced in this paper. New experiments are obviously needed to elucidate this particular point.

If stretching of the surface coat gives rise to an increase in permeability to oxygen, one might expect that the stretchings of the coat occurring during the preparatory phases of mitosis might cause a temporary increase in the oxygen uptake. Such rhythmic oxygen uptake, synchronized with the early mitotic divisions have been demonstrated (Brachet, 1945; Zeuthen, 1946). Of the two possible interpretations of the results mentioned by the authors, variations in oxidation intensity or increase of permeability to oxygen, the present hypothesis thus decides in favour of the latter.

Any conditions which influence the properties of the coat may lead to changes in the pattern of gastrulation. In Ca-free or hypertonic solutions the permeability of the membrane is increased, and in such media a high frequency of exogastrulation is observed (Holtfreter, 1933). The mechanism involved seems to be that, because of the increased permeability, oxygen may be supplied at an increased rate all around the egg, leading to simultaneous invagination along the entire marginal zone, thus giving rise to an exogastrula. The appearance of accessory organizers and other developmental anomalies in overripe eggs (Witschi, 1934; Briggs, 1941), which according to Holtfreter (1948) is a result of deterioration of the surface coat, is also easy to understand in the light of the present hypothesis.

It seems important to see whether the morphogenetic effects of lithium and thiocyanate may be incorporated in the present hypothesis. These ions may exert what may appear quantitative effects, in that treatment with lithium may lead to microcephaly, and treatment with thiocyanate to macrocephaly (see the review by Ranzi, 1953). Lithium is known to increase, and thiocyanate to decrease the viscosity of protein solutions, and it would be tempting to explain the above-mentioned morphogenetic effect by assuming that the permeability of the coat is decreased and increased, respectively, by these ions. The action of thiocyanate on the coat does not seem to have been investigated, but certain observations may be found concerning the action of lithium. Thus this ion counteracts the formation of the grey crescent (Dollander & Laurent, 1952), apparently in support of the above explanation. Sodium citrate has the same effect, although it has none of the morphogenetic effects of lithium. This need not invalidate the suggested mechanism, because lithium and citrate have quite different actions on the mechanical properties of the coat (Dollander & Labadie, 1952). By exposure to high concentrations of lithium the coat is easily destroyed (Dollander & Laurent, 1951). It may well be that the quantitative morphogenetic effects of lithium and thiocyanate are exerted through their action on the mechanical properties of the coat, although more experiments are needed to prove this. It seems certain, however, that lithium at least has more specific maybe qualitative effects. These may be exerted by suppression of the synthesis of proteins as suggested by Gustafson (1950).

CONCLUSION AND SUMMARY

A hypothetical interpretation has been advanced to explain the physiological mechanism operating in the determination of the bilateral symmetry in amphibian embryos. The basic assumption of the hypothesis is that the establishment of the dorso-ventral polarity consists in creating such conditions that oxidative processes may go on at a higher rate in one side of the egg. Of the three factors necessary, glycogen, mitochondria, and oxygen, the two former are found in the cytoplasm of the animal hemisphere. Very little is known about the localization of these components, but presumably they are distributed with radial symmetry. The third factor, oxygen, must enter through the cortex. It is known that the protein coat of the amphibian egg has a very low permeability, and it is suggested that the rate of oxygen supply is limited by the diffusion resistance of this coat. The grey crescent is assumed to represent a region of higher permeability, formed by stretching of the coat. A considerable number of observations may find a simple explanation in the light of this interpretation. It is not possible to enter upon a discussion of the applicability of the hypothesis to other species. It should only be mentioned that the importance of mitochondria in morphogenetic processes, and the question of the surface as a diffusion barrier for oxygen have been investigated in relation to the early development in sea-urchin embryos (see Gustafson & Lenicque, 1952; Lindahl & Öhman, 1938; Lindahl, 1940). Elements of the hypothesis have thus for some time been commonplace in sea-urchin studies. To apply the hypothesis directly to echinoderms meets with some difficulties, but it remains to be tried whether it is possible to overcome them.

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The Formation of the Second Maturation Spindle in the Eggs of *Limnaea*, *Limax*, and *Agriolimax*

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WITH TWO PLATES

INTRODUCTION

EGG maturation in *Limnaea stagnalis* has been described by Raven (1945, 1949). It exhibits some peculiarities, by which the *Limnaea* egg seemed to stand apart from all other animal eggs in which the maturation processes have been accurately studied so far.

In the first place it appeared that the second maturation spindle arises by a direct and gradual transformation of the centrosphere at the inner end of the first maturation amphiaster, remaining in the egg after the extrusion of the first polar body.

Secondly, the above-mentioned investigations revealed the still more astonishing fact that the inner aster of the second maturation spindle is none other than the sperm aster, which arises quite independently round about the time of the extrusion of the first polar body, grows considerably in size while the second maturation spindle is being formed, and then fuses secondarily with the latter's deep end. After the extrusion of the second polar body the aster becomes free again. It shifts to a more central position in the egg, where it remains visible for some time, but gradually disintegrates, and disappears long before the prophase of first cleavage.

Although our previous investigations, executed on a sufficiently large number of eggs, admitted of no other interpretation of the facts, this course of events appeared to be so much at variance with all descriptions given in the literature on maturation in other species that further confirmation was felt desirable. It appeared probable that an accurate study of the maturation process in eggs of related species might contribute to a better evaluation of the events found in *Limnaea*. One could hardly expect that such a fundamental process as matura-

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tion would occur in completely different ways among representatives of the same taxonomic group as, for example, the Pulmonate Gastropods.

The process of egg maturation in *Limax flavus* L. and *Agriolimax* (*Deroceras*) *reticulatus* (Müller) was therefore studied by two of us (J. A. Leussink and Miss F. C. M. Escher). Special attention was paid to the formation of the second maturation spindle, a process for which Mark's (1881) statement 'it has been customary for the second archiamphiaster to receive only a hasty description' is still valid. It appeared that in both species this spindle arises by transformation from the substance of the centrosphere, as it does in *Limnaea*. However, nothing could be detected in these species resembling the secondary connexion between second maturation spindle and sperm aster found in *Limnaea*.

It was decided, therefore, to take up once more the study of maturation in *Limnaea*, on a still more extensive and accurately timed material of eggs fixed between the telophase of the first and the end of the second maturation division. In order to facilitate the analysis of the time relations between the various components of the process, development was slowed down by keeping the eggs at a lowered temperature. This re-investigation was done by W. M. Herrebout. It gave in the main a full confirmation of previous findings, and in certain points furnished a valuable addition to them.

MATERIAL AND METHODS

(*a*) *Limnaea stagnalis*

The eggs of *Limnaea* were obtained in the usual way by stimulation with *Hydrocharis* (Raven & Bretschneider, 1942), from snails which had been raised under rather equable conditions in the laboratory from eggs laid the previous year. Oviposition took place in water of 22° C. When an egg-mass had emerged from the genital aperture for about two-thirds of its length it was seized with forceps and carefully drawn out. It was then transferred to tap-water at a temperature of from 11 to 13° C.

The rate of development at this temperature was studied in a number of egg-masses. The first polar body was extruded in eggs in the front part of the egg-mass after 30–65 minutes, in the hind-part of the egg-mass after 56–80 minutes. The period between the extrusion of the first and the second polar body amounted on an average to $86{\cdot}4 \pm 7{\cdot}8$ minutes. First cleavage began 270–310 minutes after oviposition. If these times are compared with those mentioned by Raven (1945) for eggs developing at about 20° C., it is evident that development is indeed slowed down at the lower temperature, although not very much.

Each egg-mass was divided into 6–8 parts. In each lot the moment of extrusion of the first polar body was established in as many eggs as possible. A certain number of minutes later these eggs were decapsulated and fixed in Bouin's fluid. In this way each egg-mass provided a series of successive stages of the maturation process.

The eggs were cut in 7·5 μ thick sections, which were stained with iron haematoxylin and erythrosin.

Ten egg-masses have been used, and 310 eggs have been studied in sections. They had been fixed at 5-minute intervals between 0 and 120 minutes after the extrusion of the first polar body. As the differences in stage of development reached at a certain point of time are rather considerable, especially between eggs from different egg-masses, the various lots overlap to a large extent.

(*b*) *Limax flavus*

The slugs, partly collected in cellars, partly raised in captivity, were cultured in covered glass aquaria. On the bottom there was a layer of pebbles, about 10 cm. high, which was kept almost entirely under water. A layer of leaf-mould, slanting to one side, was put on top of this gravel layer. In the deepest part there were some dry lime-tree leaves. From time to time the surface was sprinkled with water. The food, consisting of rolled oats and macaroni soaked in water, supplemented by brewer's yeast powder, was placed on the higher part of the bottom. The faeces and food remains must be regularly removed, and care must be taken that the soil is not too moist in order to prevent the growth of moulds.

The aquaria were placed in a dark cellar, but were lighted in the day-time by means of a lamp. The temperature varied in general between 15° and 18° C., but occasionally fell to 12° C. in the morning.

Egg-laying begins as a rule in the early morning. The eggs are laid in the moist part, mostly under leaves. They are deposited one by one, in a string, with an interval of from 10 to 20 minutes between successive eggs, which is nearly constant for each egg-mass. The number of eggs per batch varied from 4 to 74; as a rule there are from 20 to 40 eggs.

In order to obtain eggs for study an animal in the act of laying was transferred to a Petri dish with mould. The eggs were taken away one by one immediately after deposition, rinsed in distilled water, and dried by rolling on filter-paper. They were then placed in a Petri dish on a bottom of 2 per cent. agar, on which they developed at a temperature of 15–18° C. The first polar body was extruded 55–100, averaging 75, minutes after oviposition; the second polar body 100–35, averaging 115, minutes after the first. Eggs were fixed at different times after the formation of the first polar body; this interval was determined as accurately as possible for each individual egg.

The egg of *Limax flavus* with its envelopes is lemon-shaped, and measures about $7\frac{1}{2}$ by 5 mm. It has a firm, laminated outer capsule, which can easily be removed with a lanceolated needle. The inner capsule is a delicate membrane surrounding the viscous capsule fluid, that contains the egg-cell. The latter has a diameter of about 150 μ.

In order to isolate the egg for fixation the outer capsule is removed, and the inner capsule is pricked carefully at a point as distant from the egg-cell as possible. The capsule fluid flows out, taking the ovum along with it. To free the

egg from the fluid, a drop of sodium thioglycolate ($CH_2SH.COONa$) is added. Within 1 or 2 minutes the egg-cell comes free, and can be sucked up with a braking pipette. After brief rinsing in distilled water the egg is ready for fixation. Fixation, sectioning, and staining was as with *Limnaea*.

In total, 102 eggs, fixed at regular intervals between 26 and 137 minutes after the extrusion of the first polar body, have been studied in sections.

(*c*) *Agriolimax reticulatus*

The methods of culture and of obtaining and preparing the eggs for study resemble in general those described above for *Limax*. The slugs were fed with rolled oats, lettuce, potato slices, and fresh vegetable waste.

The eggs are laid singly, with regular intervals of about 3–5 minutes between successive eggs. The number of eggs per batch varied from 6 to 35. Freshly caught slugs produce in general more eggs than slugs kept for some time in the laboratory.

The egg in its capsules measures about 4 mm.; the egg-cell has a diameter of about 85 μ.

The first polar body was extruded about 100 minutes after oviposition at 16° C.; at 19° C. this period lasted only 50 minutes. Likewise, the time between the extrusion of first and second polar body is about 110–20 minutes at 16°, but 90 minutes at 19° C.

About 90 eggs, fixed between 0 and 120 minutes after the extrusion of the first polar body, were studied in sections.

RESULTS

Every investigation in cytology, in which the normal course of events is reconstructed from sections, which are to be arranged in a certain order and interpreted as instantaneous views of the process in question, is open to the criticism of partiality. Preconceived opinions may to a large extent determine the order in which the preparations are arranged. The investigator is tempted to lay undue stress on any picture fitting into a more or less diagrammatic representation of the course of events, and to dismiss all those cases that are conflicting with it.

In order to preclude such a delusion it is important to have an objective criterion, by which to establish the actual sequence of the pictures. We had hoped that the method of collecting the material, in which the time of fixation with respect to the moment of first polar body formation is accurately known for each egg, would provide us with such a criterion. A description of the eggs in their order of fixation would then give as near an approach to the normal sequence of events as possible.

However, it appears that this expectation has only to a small extent been fulfilled. In consequence of the considerable variations in stage of development

reached among eggs fixed at the same maturation age, and the resulting overlap of the successive lots (which may, at least in the case of *Limnaea*, be estimated as amounting to about 10 to 20 minutes), the arrangement of the sections solely using the criterion of age gives a rather confusing picture of the real process. Therefore, we still are obliged to have recourse to personal interpretation, whereby the fixation age of the eggs only provides us with a rough framework.

The above-mentioned variability in stage of development is least pronounced in *Agriolimax*. As a matter of fact, here the various lots, fixed at successive points of time with respect to the extrusion of the first polar body, hardly overlap in their development. Therefore we shall begin our description with this form.

(*a*) *Agriolimax reticulatus*

In the eggs fixed at 0 minutes the first polar body is just being constricted off. The first maturation division is in late anaphase. The spindle reaches with its outer half into the polar body, passing through the narrow 'neck' still connecting the latter with the egg. The inner end of the spindle is provided with a large aster. This has a distinct centrosphere, which shows a rather sharp boundary with the surrounding astral radiations. The centrosphere has a peculiar shape: it is elongated in a transverse direction and dumbbell-shaped, as if it were in the act of dividing. Two extremely minute granules are seen in its interior, lying at a small distance from one another on either side of the shallow constriction in the middle of the centrosphere. These granules, around which in some cases a somewhat clearer spherical area may be faintly indicated, apparently represent the centrioles. A similar dumbbell-shaped centrosphere, containing a pair of centrioles, is found in the polar body. The dyads are lying near the ends of the spindle, close to the centrosphere, but not yet against its surface.

Some other eggs, fixed between 0 and 10 minutes, are slightly further advanced. They are in telophase. The polar body has been completely constricted off, but is still connected with the egg surface by a 'mid-body', which has formed from the middle part of the interzonal region of the spindle. The dyads have now reached the centrosphere, and closely apply themselves against its outer surface in a tightly packed group. The centrosphere is still dumbbell-shaped, but its centrioles have moved somewhat farther apart, and are now lying near the centre of each of its halves (Plate 1, fig. A).

In the eggs fixed at 13–15 minutes, the mid-body and spindle remnant are still visible. The dyads are still closely applied against the centrosphere. The latter has now changed its shape. The constriction in the middle has disappeared, so that the centrosphere has become elliptical, its long axis being parallel or oblique to the egg surface. The centrioles now occupy a position about corresponding to the focal points of the ellipse. They have become more conspicuous than at previous stages, and appear as small globules with a great affinity for iron haematoxylin.

In the eggs fixed between 20 and 30 minutes the spindle remnant has disappeared. In other respects the eggs resemble those of the previous group. Particularly, the centrosphere with its centrioles shows the same relationships. But towards the end of this phase (27–30 minutes) there is a faint indication of short radial fibrils in the area of the centrosphere, starting from each centriole (Plate 1, fig. B).

This becomes much clearer in the subsequent minutes, distinct small asters being formed around the centrioles. At first the latter are still lying inwards from the extremities of the centrosphere (Plate 1, fig. C), but with the farther extension of the astral radiations the centrioles come to lie at the ends of the centrosphere. At the same time a longitudinal striation appears in the latter, transforming it into the early second maturation spindle, which is provided from the outset with equal asters at both ends. The latter are centred around the centrioles, from which they presumably have been formed. As the old radiations of the original aster, although much reduced, have not yet entirely disappeared when the new asters arise, the possibility remains that some of its astral rays are taken up in the latter.

This process has been about completed at 40 minutes. The second maturation spindle is at first parallel or oblique to the surface, but soon it rotates and places itself at right angles to the surface. Already at 45 minutes in most eggs the spindle has reached the latter position.

When the second maturation spindle has been formed, the dyads are at first still lying as a compact group against one of its sides (Plate 1, fig. D). But at 45–48 minutes they begin to invade the spindle area. Then they distribute themselves in a rather irregular way in the middle region of the spindle (Plate 1, fig. E). The 'pro-metaphase' stage thus reached lasts for a long time. At 57 minutes all eggs have reached this stage. At 62, 66, and 72 minutes the dyads have retained their irregular distribution about the spindle, then they begin to align in the equatorial plane, and at 80 minutes the metaphase is reached (Plate 1, fig. F).

In the meantime the spindle has grown considerably in length, the distance between the poles having increased about $1\frac{1}{2}$ times from the stage of fig. D to that of fig. F. Moreover, a centrosphere has formed in the asters of the second maturation spindle. In the deep aster it appears somewhat earlier and is often somewhat larger than in the superficial one. A vesicular structure is often found in its centre.

The further development takes place in the usual way. Early anaphase is reached at 95 minutes. The spindle at this stage connects with the egg surface at the animal pole, where a slight bulge is formed, in which the superficial aster flattens against the surface. At late anaphase to telophase a conical projection occurs at this place, which is then constricted off as the second polar body (117–20 minutes). The deep aster remaining in the egg has a large centrosphere.

A sperm aster has in no case been observed in the eggs of *Agriolimax reticulatus*.

(*b*) *Limax flavus*

The youngest eggs studied in sections were fixed 26 minutes after the extrusion of the first polar body. In these eggs, and in most eggs of the subsequent lots up to a fixation age of 45 minutes, the polocyte is still connected with the egg surface by a conspicuous dark 'mid-body'. A large aster beneath the egg surface at the animal pole represents the former deep aster of the first maturation spindle. It has a round or slightly oval centrosphere of moderate size, which is rather dense and deeply stained. The dyads, remained in the egg at the first maturation division, form a compact group at the boundary of the centrosphere on its side turned towards the surface. They are connected with the mid-body by the spindle remnant.

In most eggs a pair of small dark globular centrioles are found in the centrosphere of the maturation aster. As a rule they are situated at some distance from one another, more or less symmetrically with respect to the centre of the centrosphere. When the centrosphere is ovoid in shape, its long axis coincides with the line connecting these bodies (Plate 1, fig. G).

In other eggs of the same lots (35–45 minutes) the spindle remnant and mid-body have disappeared. The aster is still large, but its astral radiations show a beginning disintegration. At the same time the centrosphere has increased in size, and its stainability has markedly diminished (Plate 1, fig. H). The dyads still form a single group on its outer surface, although they may be somewhat less crowded than before.

At 45–48 minutes the centrosphere begins to elongate, often in a transverse or oblique direction. The centrioles have in the meantime moved still farther apart, and now occupy the ends of its long axis (Plate 1, fig. J). At the same time a longitudinal striation appears in the centrosphere. In this way it is transformed into the early second maturation spindle. Small asters are formed in the surrounding cytoplasm at its two ends, starting from the centrioles lying there. The old radiations of the original aster have in the meantime been further reduced, but remnants of it are still visible at the extremities of the spindle, when the new asters make their appearance. The behaviour of the dyads is very characteristic. At first they remain in their original location on the animal side of the developing spindle. Depending on the direction of its elongation, they may either be situated against one side or near one of the ends of the spindle. Once the spindle has got its characteristic shape, however, the dyads are always found in a single compact group against one side near the spindle equator.

This stage is reached at 55–60 minutes (Plate 1, fig. K). The position of the spindle is very variable. It is either parallel, oblique, or perpendicular to the surface in about equal numbers of cases; apparently the direction of its elongation has been quite haphazard. In subsequent stages the number of spindles with their long axis at right angles to the surface gradually increases. But as late as the pro-metaphase (84–88 minutes) the second maturation spindle may still be

in a nearly tangential position, and even early anaphase spindles may occasionally still be slightly oblique to the surface.

The small asters at the spindle ends soon enlarge. Up to this stage they were equal in size, but now the aster nearest the surface lags behind the other in its growth. In the deep aster a clear central area soon appears, whereas a centrosphere is lacking or remains very small in the outer aster.

The dyads, which were still situated outside the developing spindle against one of its sides, now begin to penetrate into it (75–76 minutes). This can be seen with extraordinary clearness in sections in which the spindle is cut transversely (Plate 1, fig. L). The process is apparently rather a slow one, for at 84–88 minutes it has not yet been completed, the dyads still showing an irregular distribution within the spindle. Their regular arrangement into an equatorial plate is reached between 91 and 104 minutes (Plate 1, fig. M). As in *Agriolimax*, a marked growth in length of the spindle takes place between the stages of fig. K and fig. M. Now mitosis rapidly continues. At 105–9 minutes all eggs are at early anaphase. The spindle has made contact with the egg surface at its outer end. The aster at this end becomes still more reduced. A small indentation of the egg surface may occur at the point of connexion. The inner aster has a big, clear centrosphere. Then a conical projection of the egg surface appears, and the second polar body is constricted off. This has been accomplished at 114–17 minutes. A conspicuous mid-body and spindle remnant connect the polar body with the egg for some time. The chromosomes retain their compact structure at first after the extrusion of the polar body, but at 134–7 minutes they have begun to swell into karyomeres. The latter are less conspicuous than they are, for example, in *Limnaea*, and apparently soon unite into a polymorphic female pronucleus.

The deep maturation aster, remaining in the egg when the polar body is extruded, remains visible for another 20–30 minutes. It is possible that it even increases somewhat in diameter during this period. At the same time, however, its astral radiations become blurred from their inner end outwards, so that its central area (centrosphere) increases enormously in size, meanwhile showing signs of a moderate vacuolization.

A sperm aster has first been found in these eggs at about 40 minutes. In all younger eggs it is lacking, whereas most eggs of later stages contain a distinct sperm aster. As a rule it is a small structure (about 10 to 15 μ) without centrosphere or distinct centriole, but occasionally it is larger and has a centrosphere. In some cases, moreover, the sperm aster is more or less dicentric, varying from a slight reduplication of its central part, to two separate asters a small distance apart. In total, 6 such double sperm asters have been observed against 38 single ones. Neither especially large nor dicentric sperm asters are restricted to certain periods; they occur both early and late. The sperm aster shows no definite relationships to the second maturation spindle. After the latter has been completely formed the two structures are found together in the same egg. No significant growth of the sperm aster occurs during the early stages of the second

maturation division. As soon as the second polar body is extruded, however, a rapid growth of the sperm aster sets in. At the same time a clear centrosphere is formed in its centre, which enlarges with even greater rapidity, and soon becomes strongly vacuolized. Ten minutes after the extrusion of the second polar body (124–6 minutes) only a large spherical vacuolated area represents the former sperm aster; after a further 10 minutes this has disappeared too, though a vaguely delimited, somewhat clearer area in the cytoplasm may still indicate its former position.

(*c*) *Limnaea stagnalis*

As it was our objective to put to the test the interpretation of the maturation process in *Limnaea stagnalis* given by Raven (1949), we have, in our study of the eggs of this species, assiduously looked for data conflicting with this view. It must be said, however, that we have in the main points not been able to come to a different conclusion, so that the previous interpretation seems the only one which can in any reasonable way be made to fit the facts. On the other hand, this study has afforded some supplementary data which provide a clue for the explanation of the aberrant course of the maturation process in the *Limnaea* egg.

In some eggs, fixed at 0 and 10 minutes, the later phases of the first maturation division are seen. The aster at the inner end of the maturation spindle is large, and has a big clear centrosphere (Plate 1, fig. N). In some eggs this area has a dark centre, or exhibits alternate concentric darker and lighter zones. These pictures are very inconstant and variable, however, and probably are mainly due to accidental variations in the state of fixation.

With the formation of the first polar body as a conical to hemispherical protrusion of the egg surface the maturation spindle is taken along upwards, so that its inner aster comes to lie nearer the surface than before. The central group of dyads, which has reached the margin of the centrosphere at the end of anaphase, therefore lies at a little distance beneath the surface when the polar body is constricted off (Plate 1, fig. O). The spindle remnant remaining in the egg soon becomes blurred and disappears, but the aster, with its centrosphere, remains.

A sperm aster is found for the first time in some eggs at 20 minutes, in which the first polar body has just been extruded. At its first appearance the sperm aster is a very small structure, measuring about 5 μ in diameter. Apparently it grows rapidly in size, however, as in other eggs of the same age-class there are larger sperm asters, measuring 10 μ in diameter or more. The early sperm aster has no centrosphere; the astral rays meet in one point at its centre. It lies near the centre of the egg, or shifted somewhat towards one side, but never quite near to the egg surface. It shows no distinct topographical relations to the sperm head which has remained behind as a compact dark body lying immediately beneath the egg surface. In most cases the sperm aster is far removed from the maturation aster, but occasionally the two structures lie fairly close to each other.

In a group of eggs fixed 20–35 minutes after the extrusion of the first polar body, the last remnants of the first maturation spindle proper have disappeared. The inner aster is still well developed, however, and has a big, nearly spherical, centrosphere. In comparison with the previous stage its astral radiations have become shorter and are less distinct. The centrosphere, on the contrary, has markedly increased in size; its diameter is, on an average, about 16 μ at this stage. It is still lightly stained and more or less structureless. It is capped on its outer side by the dyads, which have remained on its surface (Plate 1, fig. P). When they first reached the margin of the centrosphere the dyads formed a rather tightly packed group. Now, beginning at about 20 minutes after the extrusion of the polar body, they move a little apart in all directions, along the outer surface of the centrosphere, until they are arranged in a more or less regular ring.

A careful study of the eggs at this stage shows that in many cases a small globular body has become visible in the spherical centrosphere. It lies near the latter's centre, or somewhat eccentrically in its outer half. It has only a moderate affinity for iron haematoxylin. The regular occurrence of these bodies, their rather distinct appearance and more or less fixed place, and a comparison with similar bodies found in *Limax* and *Agriolimax*, have convinced us that we have to do here with a true centriole. Contrary to previous statements (Raven, 1949) the egg cytocentre of *Limnaea* therefore contains a centriole indeed. It must be emphasized, however, that it is always single at this stage, contrary to the relationships in other Pulmonates, where it has already divided during the first maturation division. Notwithstanding careful search, never more than one centriole has been found in *Limnaea* at this stage or in any of the subsequent stages of maturation.

The sperm aster is on an average somewhat larger than before, reaching a diameter of about 12 μ. In some eggs it lies rather close near the inner side of the maturation aster (Plate 1, fig. P). In one egg it is still connected with the end of a disintegrating sperm tail, proving that it represents the sperm aster indeed.

A group of eggs, varying in fixation age from 20 to 40 minutes, shows the gradual transformation of the centrosphere of the first maturation aster into the second maturation spindle. Its stainability increases. At the same time it begins to elongate about perpendicularly to the surface. At first it has the shape of a regular rotation ellipsoid. The single centriole now seems to occupy one of the 'focal points' of the ellipsoid, the other one being empty (Plate 2, fig. A). Apparently this stage is of an extremely short duration; it is represented by only two eggs in our material. Soon the centrosphere becomes more or less egg-shaped, one end being pointed and the other end blunt. The pointed extremity, which is the one near the centriole, is always directed towards the surface. The centriole in the meantime moves to this extremity of the centrosphere. Already before it has reached it a few short radial fibrillae may be sketchily indicated in the substance of the centrosphere around it (Plate 2, fig. B). When it has arrived

at the pointed end of the centrosphere, however, it forms the starting-point of astral radiations which begin to extend into the neighbourhood, in this way forming the outer aster of the second maturation spindle (Plate 2, fig. C). The other (blunt) end of the spindle, on the contrary, is only surrounded by an indistinct fringe of disappearing astral rays of the original first maturation aster. In spite of careful search in no case has a new centre of radiation been found at this pole.

In the centrosphere a longitudinal striation appears, indicating a parallel orientation of the cytoplasmic micellae. It begins at the pointed extremity, where the centriole is situated, and extends gradually into the outer half of the centrosphere. The other half, which widens still more, at first retains its original appearance (Plate 2, fig. C); only gradually the longitudinal striation extends into this half, transforming in this way the centrosphere into the second maturation spindle. Even then, however, this spindle is quite asymmetric, its inner end lacking an aster and showing a blunt rounded outline. The dyads, which at first surrounded in a loose circle the outer end of the centrosphere, gradually move on along the surface of the developing spindle until they have reached its equatorial region (Plate 2, figs. A–C).

In the meantime the sperm aster, which has further increased in size and begins to show a clear area in its centre, has still further approached the inner end of the developing second maturation spindle. Especially in the latter part of this period the two structures are found in many eggs near together, either in the same or in consecutive sections. When the sperm aster has come quite near the inner end of the spindle, it becomes somewhat asymmetric, the astral radiations on the side turned towards the spindle being shorter than on the opposite side (Plate 2, fig. D).

The next stage is formed by a group of eggs of from 30 to 65 minutes' fixation age. At first sight it appears that they are separated by a wide gap from those of the previous group in so far as they all show a well-developed second maturation spindle, provided with distinct asters at both ends. If we pay attention to the position of the dyads, however, which are still arranged in a loose irregular ring against the outer surface of the spindle near or slightly above its equator (Plate 2, fig. F), as they were towards the end of the previous period, it is evident that these eggs cannot be much farther advanced. Only in the oldest eggs of this group the dyads begin to penetrate into the spindle, where they arrange themselves into a metaphase plate (Plate 2, fig. H).

There is a remarkable difference between the two ends of the second maturation spindle (Plate 2, figs. F, G). The outer end is still more or less pointed. The aster at this end is small, and has in no case a central clear area. The spindle fibres converge towards the centre of the aster, where the centriole may still be seen as a vaguely delimited dark body. The inner aster, on the contrary, is much bigger and has a large clear centrosphere. Very striking is the transition between the spindle and the inner aster. The spindle is hardly narrowed at this end. The

spindle fibres maintain a more or less parallel course, until they end at the boundary of the centrosphere (Plate 2, fig. E).

A further peculiarity of this stage is the sudden disappearance of the sperm aster as an independent structure. Neither in this nor in any further stage until the extrusion of the second polar body, has an isolated aster independent of the maturation spindle ever been found.

If we take together (1) the sudden appearance of a large aster with well-developed centrosphere at the inner end of the spindle, which was without anything resembling an aster at earlier stages; (2) the equally sudden disappearance of the sperm aster, which was found as an independent structure near the inner end of the maturation spindle at the end of the previous period; (3) the remarkable shape of the maturation spindle, especially of its transition with the inner centrosphere, there is, in our opinion, but one unforced explanation of these findings —the sperm aster and maturation spindle have fused into one functional entity.

There is one further particular in the eggs of this period which deserves special attention. In the region of transition between the maturation spindle and its deep aster there is a zone of material, which stains somewhat more deeply than its surroundings, and contains numerous dark granules and small vacuoles (Plate 2, figs. E–H). A similar zone is indicated already at the end of the previous period between the deep end of the spindle and the sperm aster, as soon as the latter has approached the spindle to within a short distance (Plate 2, fig. D). These pictures suggest that the fusion of the sperm aster with the deep end of the maturation spindle is accompanied by a certain cytochemical activity in the zone of fusion.

The further history of the second maturation division may be followed in the eggs fixed between 60 and 120 minutes. It offers no further peculiarities. The maturation spindle comes into contact with the surface at the animal pole, where a small indentation is temporarily formed (Plate 2, fig. H). At the end of anaphase the formation of the polar body begins with a hemispherical protrusion (Plate 2, fig. J). After the second polar body has been pinched off the deep aster (i.e. the former sperm aster) becomes free again and shifts to a somewhat deeper level (Plate 2, fig. K). Its centrosphere increases considerably in size and shows a rapid vacuolization. It remains for some time as a sharply delimited vacuolated sphere, surrounded by an annular zone, in which the astral radiations are at first clearly visible, but soon become blurred and vanish altogether (Plate 2, fig. M). The chromosomes begin immediately after the extrusion of the second polar body to swell into karyomeres, which leave their position at the boundary of the centrosphere and assemble immediately beneath the egg cortex at the animal pole. Here they are soon accompanied by the sperm nucleus, which likewise has begun to swell immediately after the extrusion of the second polar body. At the same time it migrates towards the egg karyomeres at the animal pole, which it may have reached already 20 minutes later (Plate 2, fig. L).

DISCUSSION

1. It is a noteworthy fact that our time sees a revival of morphological thinking in cytology. In the early years of cytological research, when the elaboration of a suitable microscopic technique had opened up vast new fields of study, the various components of the cell, as they appeared in microscopic sections, were assiduously studied and described in every possible detail. Particularly, much attention has been paid to the structure of the 'achromatic apparatus' of the meiotic and mitotic divisions. In the years around 1900 we find very detailed descriptions of the structure of spindles, asters, and 'centrosomes' in the papers dealing with maturation and early cleavage of animal eggs. Obviously, the idea that a morphogenetic process such as cell-division can only be understood on the basis of an accurate knowledge of the morphology of all structures involved, is at the root of these investigations.

A reaction to this morphological outlook occurred in the following decades. With the rise of colloid chemistry and cell physiology, a more physiological attitude became predominant. It became customary to look with great reserve at the many intricate structures described by an earlier generation of cytologists, and to dismiss most of them as fixation artifacts and coagulation products in the colloid mixture which is the protoplasm. The reality of such structures as spindle fibres, astral rays, and centrosomes was questioned by many authors.

But the pendulum has already passed its culminating point, and in the last few years it is evident that a new morphological era is rapidly advancing. This is especially due to the establishment of new methods of cytological study, such as phase contrast and electron microscopy. These methods have already in many cases furnished definite proof, not only of the reality of certain cytological components, but of an intricacy of their fine structure of which we had not the slightest idea before. We have come again to look at cell organelles like mitochondria, Golgi apparatus, &c., as tools serving special functions in cell life, and showing a high degree of organization adapted to that end.

It is therefore not surprising that in these years the 'achromatic apparatus' of the classical authors has also risen to new esteem. The reality of spindle fibres, astral rays, and centrioles is no longer in doubt (Schrader, 1953). As regards the latter, De Harven and Bernhard (1956) have not only found this body in electron-microscopic study of ultrathin sections of various vertebrate tissues, but have even shown that it has a characteristic shape and ultrastructure, which makes it well suited as an organel for the production of protein fibres.

In the light of these facts a renewed study of some puzzling aspects of the maturation divisions in Pulmonates seemed appropriate. Previous investigations had shown the aberrant course, especially of the second maturation division, in *Limnaea stagnalis*. If this could be confirmed the question arose in what way this atypical case could be reconciled with the prevailing views on the role played by the centres in the establishment of the spindle apparatus.

2. Our observations have clearly shown that in the three species studied the second maturation spindle arises by direct transformation from the centrosphere of the deep aster of the first maturation spindle. Apparently this is generally the case in the Pulmonata. The descriptions and illustrations given by Garnault (1888–9, *Helix*), Kostanecki & Wierzejski (1896, *Physa*), Byrnes (1900, *Limax agrestis*), Linville (1900, *Limax* and *Limnaea elodes*), and Lams (1910, *Arion*) all point to the same manner of origin of the second maturation spindle in these species, although many of these authors do not say so explicitly.

As a rule the centriole at the deep end of the first maturation spindle has divided at a certain stage. In *Arion* this may, for example, occur already at the prophase of the first maturation division. Some time after the extrusion of the first polar body the two daughter centrioles begin to move apart within the area of the centrosphere. At the same time the latter elongates and becomes elliptical, with its long axis coinciding with the line connecting the centrioles. Some connecting fibres between the centrioles have been interpreted as the beginning of a 'central spindle' (e.g. *Physa*, *Arion*). The main mass of the spindle arises, however, by the appearance of a longitudinal striation in the substance of the centrosphere, occurring at the time when the centrioles have reached the ends of the long axis of the centrosphere. This striation is probably due to a fibrillar orientation of the cytoplasmic micellae.

The formation of the second maturation spindle in *Limnaea stagnalis* does not conform to this general scheme. In previous investigations no centriole has been found in these eggs (Raven, 1945, 1949). A careful renewed study has now shown that a centriole is present indeed, but that it is always single. Although similar granules had occasionally been seen in our previous investigations, they had always been considered as coagulation products or cytoplasmic granules accidentally present in this region, the more so as a true centriole was expected to be paired at this time. But its regular occurrence and gradual displacement toward one of the poles has now convinced us of its real nature.

When the centriole becomes first visible in *Limnaea* it lies near the centre of the centrosphere, but soon it shifts towards that pole of the elongating centrosphere, which is nearest the surface. At the same time the centrosphere, which is at first a regular rotation ellipsoid, becomes egg-shaped, the outer pole containing the centriole being pointed and the other end blunt. The longitudinal striation, indicating the orientation of cytoplasmic particles and the formation of spindle fibres, neither here occurs simultaneously throughout the centrosphere nor begins from both ends, as in the other species, but it begins only at the pointed extremity, where the centriole is situated, and extends gradually into the outer half of the centrosphere, the inner half at first retaining its original appearance. Only gradually the striation extends into this half, too, whereby the whole centrosphere is transformed into the second maturation spindle, which is, however, not symmetrical, as in the other species, but quite asymmetrical, its inner end still being blunt and lacking an aster.

3. As regards the orientation of the developing second maturation spindle, there is a marked difference between the three species studied. In *Agriolimax reticulatus* the first elongation of the centrosphere always seems to take place parallel to the egg surface. As a matter of fact, already at late anaphase of the first maturation division the centrosphere is dumbbell-shaped, with its long axis in a transverse direction. When the centrioles move apart they are displaced along this axis. About 15 minutes later, when the centrosphere has rounded out and become elliptical in outline, in some eggs it has begun to rotate, and has now an oblique position with respect to the surface. In other cases the centrosphere and developing spindle retain their original position, however. Only when the spindle has been completely formed, at 40 minutes, rotation begins in all eggs, and already at 45 minutes the spindle is perpendicular to the surface in most eggs.

In *Limax flavus* the first elongation of the centrosphere apparently takes place in a haphazard direction. The early second maturation spindles are either parallel, oblique, or perpendicular to the surface in about equal numbers of cases. Their definitive orientation at right angles to the surface occurs only gradually, and even early anaphase spindles may still be slightly oblique.

As regards *Limnaea stagnalis* there is a curious difference between the eggs studied previously (Raven, 1949) and those of the present investigation. In the former the first elongation of the centrosphere took place in the majority of cases in a tangential or oblique direction with respect to the egg surface. Therefore the early stages in the formation of the second maturation spindle were mostly nearly parallel to the surface or oblique, and only when the spindle was nearly completed and shortly before it fused with the sperm aster, it turned into its definitive position at right angles to the surface.

In the eggs studied here, however, the relationships are quite different. Even the first elongation of the centrosphere takes place at right angles to the surface, so that the developing second maturation spindle has from the beginning its definitive orientation. Among forty-nine eggs fixed between the first elongation of the centrosphere and the early metaphase stage, there is not a single one where the developing spindle has its long axis approximately parallel to the surface, and in only three of them does it deviate by more than about 15–20° from the radial direction.

The cause of this difference between the two groups of *Limnaea* eggs studied in different years is not immediately clear. It is improbable that genetic differences play a part, since all eggs were from snails caught in the neighbourhood of Utrecht; the possibility that they belonged to different sub-populations cannot be ruled out, though.

On the other hand, it must be remembered that the *Limnaea* eggs in the present investigation were cooled to 11–13° C. in order to slow down development, whereas those studied previously had developed at room temperature of about 20° C. The most probable explanation of the difference in behaviour of

the two groups seems, therefore, to be that it is due to temperature. Various processes in the developing egg may be temperature-dependent to a different degree, so that shifts in the time of action of various factors may occur. Apparently at a lower temperature the directing forces bringing the second maturation spindle into its normal position begin to work at a relatively earlier stage. This is a warning that even in the description of the normal course of developmental processes the external circumstances have to be taken into account.

4. The behaviour of the dyads in the three species is related to the differences in development and orientation of the spindles. In all cases, at the end of anaphase of the first maturation division the dyads remaining in the egg have reached the margin of the centrosphere of the deep aster. They apply themselves closely against it in a tightly packed group, but never penetrate into the substance of the centrosphere.

In *Agriolimax reticulatus* the dyads remain in this position when the centrosphere transforms into the second maturation spindle. As the first elongation of the centrosphere always takes place parallel to the surface, and the dyads lie against its outer side, they are from the beginning found sideways against the spindle in its equatorial region. When the spindle turns into a radial position it takes the dyads along with it. Shortly afterwards the latter begin to penetrate into the spindle, and arrange themselves, at first irregularly, in its middle region.

In *Limax flavus* the relationships are quite similar. Since, however, the first elongation of the centrosphere does not always occur in a tangential direction, but may also be oblique or nearly radial, the dyads may at first lie near one of the ends of the developing spindle. In these cases, however, they are then displaced along the surface of the spindle, until they form a compact group on one side in the equatorial region. As in *Agriolimax* they invade the spindle area only secondarily from one side, which is very clearly seen in transverse sections of the spindle. They remain also in this case irregularly distributed in the spindle for some time, and only gradually become aligned into an equatorial plate.

Finally, in *Limnaea stagnalis* the dyads, at first forming a compact group against the outer side of the centrosphere, after some time move a little apart, so that they become arranged into a more or less regular ring. When the centrosphere begins to elongate, perpendicularly to the surface, the dyads form a crown around its outer pole. Their characteristic movement is not directly related to their position with respect to this pole, however. In our previous investigation (Raven, 1949) it was found that it also occurs even in those cases where the first elongation of the centrosphere is nearly parallel to the surface, so that the ring of dyads lies at first asymmetrically against one side of the developing spindle.

When the centrosphere lengthens more and more and its peripheral end becomes pointed, the circle of dyads moves along its outer surface until it has reached the equatorial region of the developing spindle. In this position the dyads remain for a considerable time even after the sperm aster has fused with

the deep end of the spindle. Only then do they begin to penetrate into the spindle, where they arrange themselves into a metaphase plate.

The events leading up to the formation of a metaphase spindle in the three species studied, though showing marked differences, agree in some essential points. They show with extraordinary clearness that the main mass of the second maturation spindle, including its continuous fibres, is not directly derived from the nucleus, but from the cytoplasm, notably from the centrosphere. Probably the centrioles play a part in the formation of the spindle fibres. The chromosomes, however, are situated outside the spindle area till a rather late stage. They penetrate only secondarily into the spindle and become attached to the spindle fibres. It cannot, of course, be excluded that the transformation of the centrosphere into the spindle occurs under the influence of the chromosomes lying quite near, but there is no evidence to support this view.

5. In those cases where the two centrioles within the deep centrosphere of the first maturation spindle move to opposite poles of the elongating centrosphere, as in *Limax* and *Agriolimax*, new asters are formed at both ends in the cytoplasm outside the transforming centrosphere. As a matter of fact, short astral radiations may already be indicated around the centrioles within the centrosphere area, before the centrioles have reached the ends of the centrosphere. This is the case in *Agriolimax reticulatus*, and the same has been described for *Limax* (*Agriolimax*) *agrestis* (Byrnes, 1900) and *Arion empiricorum* (Lams, 1910). But the main part of the asters is formed also in these cases after the centrioles have arrived at the poles.

The rays of the old aster surrounding the centrosphere begin to disintegrate when the latter elongates and transforms into the spindle. However, traces of the old astral rays may still be present at the moment when the new asters make their appearance. Some authors have stated that the new asters develop from parts of the old one. In our opinion this is a mere matter of words, however. The essential thing is that the oriented condition of the cytoplasmic structure of which the aster is the visible expression, begins again to spread outwards from the centrioles occupying the poles of the developing spindle.

In *Limnaea*, where the centriole after the first maturation division remains undivided, and moves as a whole towards the outer pole of the centrosphere, the aster at this pole is formed in the cytoplasm outside the centrosphere as in the other species. The inner aster, on the contrary, is supplied by the sperm aster, which has arisen independently of the spindle, but fuses secondarily with its inner end.

6. This brings us to the development of the sperm aster, which shows great differences among the Pulmonata. Sometimes it originates in the immediate neighbourhood of the sperm nucleus, in other cases it first becomes visible at some distance from the latter. In a few instances its origin can be distinctly traced back to the middle piece of the sperm, e.g. in *Physa* (Kostanecki & Wierzejski, 1896). The observation that the early sperm aster in *Limnaea*

stagnalis is sometimes still connected with one end of the sperm tail also pleads in favour of this origin. The same has been observed in *Limax flavus*. Soon the sperm aster moves away from the sperm nucleus, which for some time remains behind beneath the egg surface.

Initially the sperm aster is only small, and has no centrosphere, the astral rays meeting in one point. Later it increases in size, and a centrosphere may then appear.

The time of appearance of the sperm aster differs. In *Limnaea* it becomes visible during or shortly after the extrusion of the first polar body, in *Limax flavus* slightly later, but still before the formation of the second maturation spindle; in still other cases (*Physa*, *Bulinus*) not before the meta- to anaphase of the second maturation division.

The greatest variation obtains with respect to the further development of the sperm aster. At least five possibilities may be distinguished:

(*a*) The sperm aster divides and forms an amphiaster with central spindle, which remains in existence till first cleavage and becomes the cleavage spindle. This has been described in *Physa fontinalis* by Kostanecki & Wierzejski (1896).

(*b*) A more or less distinct division of the sperm aster takes place, leading to the formation of a dicentric aster, which disappears, however, before the prophase of first cleavage. This may sometimes occur in *Limnaea elodes* (Linville, 1900), in other *Limnaea* species (De Larambergue, 1939), and also in *Limax flavus*.

(*c*) The sperm aster remains undivided, and disappears after some time, at any rate before the prophase of first cleavage. This is the rule in *Limax maximus* (Linville, 1900) and *Bulinus contortus* (De Larambergue, 1939); the same holds for *Limax flavus*.

(*d*) The sperm aster grows rapidly in size, and a large centrosphere is formed in its centre. When the second maturation spindle has been formed, and is placed perpendicularly to the surface, the sperm aster fuses with its inner end and becomes the deep maturation aster. This has up till now only been observed in *Limnaea stagnalis*, but some figures given by Linville (1900) and Crabb (1927) suggest that it also occurs in *L. elodes* and *L. stagnalis appressa* Say.

(*e*) There is no sperm aster at all. This is obviously the case in *Agriolimax reticulatus*, at least as far as the period between the two maturation divisions is concerned. In *Limax* (*Agriolimax*) *agrestis* (Byrnes, 1900) and *Arion empiricorum* (Lams, 1910), as a rule no sperm aster has been observed either.

7. If we compare these five different modes of development of the sperm aster in the Pulmonates, it is evident that the first-mentioned one tallies best with a diagrammatic representation of the fertilization process, according to which the cytocentra forming the poles of the cleavage spindle are derived by division from

the sperm cytocentre. If the description given by Kostanecki & Wierzejski is correct, *Physa fontinalis* is the only Pulmonate known to conform to this scheme. It must be said, however, that there is some doubt whether these authors have not been influenced too much by preconceived opinions as to the course of the process. Kostanecki has given some years later (1904) a similar description for the egg of the Lamellibranchiate *Mactra*. In no other Mollusc, however, has a clearly visible material continuity between sperm cytocentre and cleavage amphiaster been found up to the present. Moreover, Kostanecki & Wierzejski mention that in *Physa*, after the pronuclei have met, the astral rays of the amphiaster decrease, and only with some difficulty may two centra be seen which are considered to be identical with the poles of the sperm amphiaster.

Accepting for the moment, however, the exactitude of these authors' description, it is evident that the possibilities (*a*), (*b*), (*c*), and (*e*), mentioned above, form a series of decreasing importance in the role played by the sperm aster, at least as regards its visible expression. It remains possible, of course, that in all these cases the genetic continuity between sperm cytocentre and cleavage amphiaster is preserved. If one considers, however, that an aster, when present, is easily observable in these rather yolk-rich eggs, because the yolk granules are expelled from the inner denser part of the aster area, it is evident that the sperm cytocentre is lacking the power to orientate the cytoplasmic micellae, at least during part of its cycle. Evidently the sperm aster is a more or less rudimentary structure in most Pulmonata. The great significance for the normal course of the fertilization process, which has been ascribed in other groups to the gradual extension of its astral radiations through the egg cytoplasm—Bataillon's 'onde de gélification'—apparently does not apply to the Pulmonates (the same holds, as a matter of fact, for the other Molluscs).

8. The case (*d*) mentioned above, hence the peculiar development of the sperm aster in *Limnaea stagnalis*, evidently is not in line with the other possibilities. Here the sperm aster, instead of remaining rudimentary and being reduced after a shorter or longer period without having played an apparent role in the processes of maturation and fertilization, undergoes a marked development, and fuses with the second maturation spindle, entirely taking the place of the deep aster of this spindle in other species. Apparently the sperm aster, having lost its original function in the fertilization process, was thereby free to take on a different task. Only after the extrusion of the second polar body does it become free again, and now disintegrates rapidly by vacuolization of its central area, in the same way as it does, for example, in *Limax*.

In trying to explain this remarkable behaviour of the sperm aster in *Limnaea*, an obvious step is to correlate it with the peculiar shape and mode of origin of the second maturation spindle in this species. In other species this spindle is symmetrical from the outset, the centrosphere from which it develops having at first approximately the shape of a regular rotation ellipsoid, and then getting the characteristic spindle shape when the centrioles have reached the poles, while

asters of equal size develop at its two ends. On the contrary, in *Limnaea* the centrosphere becomes egg-shaped when the centriole moves to its outer pole. This end becomes pointed, and an aster develops around the centriole, whereas the inner end remains blunt and rounded, and is devoid of astral radiations.

Although no generally accepted theory of mitosis exists up to the present (Schrader, 1953), it can hardly be denied that the asters play an important part in the division process. In studying the divisions of animal eggs one cannot help being impressed by their marked development. In the maturation divisions it is especially the aster at the deep end of the spindle which shows signs of a considerable activity. One is compelled to think that it must be somehow indispensable for the generation of the force driving out the polar body. Nobody who is familiar with these pictures will be prepared to believe that the normal extrusion of a polar body might be possible in the total absence of a deep aster.

However this may be, it is a matter of fact that the failing aster at the deep end of the second maturation spindle in *Limnaea* is replaced by the sperm aster. This may be a direct consequence of the lack of astral radiations at this end of the spindle, whereby, instead of the mutual repulsion between two asters, the attractive forces between aster and spindle end come into play. Once this fusion has occurred, the sperm aster may entirely take the place of the deep maturation aster, and play its part in driving out the polar body.

9. We come to the conclusion, therefore, that the primary cause of the aberrant course of the second maturation division in *Limnaea* is the failure of the egg cytocentre to divide properly. It must be kept in mind that, according to the classical view, the egg cytocentre loses its capacity to divide, and becomes inactive after the completion of both maturation divisions, and is then replaced by the sperm cytocentre in normal fertilization. We need only to assume that the inactivation of the egg cytocentre has been anticipated by one, or rather one-half, division cycle in *Limnaea*, to explain its peculiarities. The centriole remaining in the egg after the completion of the first maturation division may still play a certain role in the formation of a new spindle, but it remains single, so that an asymmetric spindle with one aster is the result.

If we take the evolutionary point of view, it is clear that a mutation bringing about such a precocious inactivation of the egg cytocentre might easily have led to an abortion or suppression of the second maturation division, followed either by disturbed development and extinction, or by polyploidy and parthenogenetic development. The parthenogenetic Prosobranchiate *Potamopyrgus jenkinsi* may have arisen in this way (Sanderson, 1940). It is only due to the fortunate coincidence that a sperm aster, free to take over the task of the failing deep maturation aster, is present at the right moment in the *Limnaea* egg that the second maturation division is completed in a normal way in this case.

There is still another side to this question. It is generally believed that a mutation, altering the normal course of development, will have the more profound effects, the earlier its action becomes manifest. If this be true, we may surmise

that the primary alteration brought about in the course of egg maturation may have deeply influenced further development in *Limnaea*. It is conceivable that a modification of development occurring at such an early stage may lie at the root of the formation of a new genus or even a higher taxonomic category. It is, therefore, important to investigate the taxonomic distribution of this peculiar mode of formation of the second maturation spindle. As stated above, there are some indications that it occurs also in other species of the genus *Limnaea*, but a renewed study of this point will be necessary. Whether other genera of the Limnaeidae, or even of other families of Basommatophora show the same peculiarity, remains for future investigations to elucidate.

SUMMARY

1. Egg maturation has been studied in *Agriolimax reticulatus*, *Limax flavus*, and *Limnaea stagnalis* with special regard to the formation of the second maturation spindle.

2. In the three species the second maturation spindle arises by direct transformation from the deep centrosphere of the first maturation amphiaster.

3. In *Agriolimax reticulatus* and *Limax flavus* there is a pair of centrioles moving apart to opposite poles of the developing spindle. In *Limnaea stagnalis* there is only one centriole, which remains undivided and moves as a whole to the outer pole of the spindle.

4. The developing spindle in *Agriolimax* and *Limax* is symmetrical and gets an aster at both ends, arising in the cytoplasm outside the original centrosphere. The developing spindle of *Limnaea stagnalis* is asymmetrical, egg-shaped, and aster formation occurs at its pointed outer end only.

5. The inner aster of the second maturation spindle in *Limnaea* is provided by the sperm aster, which fuses secondarily with the blunt inner end of the spindle.

6. There are some indications that the fusion between these two structures is accompanied by a certain cytochemical activity in the region of fusion.

7. The sperm aster in *Limax flavus* appears some time after the extrusion of the first polar body; it does not fuse with the second maturation spindle but disintegrates by vacuolization soon after the extrusion of the second polar body. In *Agriolimax reticulatus* no sperm aster has been observed.

8. In the three species the dyads apply themselves closely against the centrosphere but do not penetrate into it. In *Agriolimax* and *Limax* they lie as a compact group on one side against the equatorial region of the developing spindle. In *Limnaea* they become arranged into an irregular ring, which encircles the outer pole of the developing spindle and gradually shifts to the equatorial region of the spindle. In the three species the dyads only penetrate into the spindle when the latter has been completely formed.

9. The results are discussed with reference to the relationships in the

Pulmonates in general. It is concluded that the sperm aster is a more or less rudimentary structure in this group.

10. In *Limnaea* the sperm aster has taken on a new function in the mechanism of polar body extrusion.

11. The primary cause of the atypical development of the second maturation spindle in *Limnaea* is, presumably, the precocious inactivation of the egg cytocentre, which divides no more after the completion of the first maturation division.

ACKNOWLEDGEMENT

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EXPLANATION OF PLATES

Plate 1

Figs. A–F. Formation of second maturation spindle in *Agriolimax reticulatus*.

Fig. A. 0–10 minutes. Telophase of first maturation division. Centrosphere of deep aster dumb-bell-shaped, distinct centrioles. Dyads against outer surface of centrosphere.

FIG. B. 30 minutes. Centrosphere has become ellipsoid; centrioles moving apart. Dyads not visible in this section.

FIG. C. 40 minutes. Disintegration of old aster. Centrioles near poles of centrosphere; beginning formation of new astral radiations. Appearance of longitudinal striation in centrosphere. Dyads not visible in this section.

FIG. D. 45–50 minutes. Second maturation spindle with distinct asters, nearly perpendicular to surface. Dyads still in compact group against one side of spindle.

FIG. E. 62 minutes. Pro-metaphase stage of second maturation division. Dyads have penetrated spindle and arranged themselves irregularly in its middle zone.

FIG. F. 80 minutes. Metaphase of second maturation division. Dyads have arranged themselves in equatorial plane.

FIGS. G–M. Formation of second maturation spindle in *Limax flavus.*

FIG. G. 26 minutes. Telophase of first maturation division. Mid-body and spindle remnant. Centrosphere of deep aster with distinct centrioles. Dyads against outer surface of centrosphere.

FIG. H. 35–38 minutes. Tangential section. Centrosphere has become ellipsoid; centrioles moving apart. Dyads not visible in this section.

FIG. J. 45–48 minutes. Disintegration of old aster. Further elongation of centrosphere. Centrioles near poles of centrosphere, beginning formation of new astral radiations. Appearance of longitudinal striation in centrosphere. Dyads not visible in this section.

FIG. K. 55–60 minutes. Centrioles have reached poles, formation of new asters. Dyads still in compact group against outer side of spindle.

FIG. L. 75–76 minutes. Transverse section of second maturation spindle. Dyads still in compact group, but begin to invade spindle area.

FIG. M. 91–104 minutes. Metaphase of second maturation division. Dyads have arranged themselves in equatorial plane. Deep aster larger than superficial one, with large centrosphere.

FIGS. N–P. Completion of first maturation division in *Limnaea stagnalis.*

FIG. N. 0 minutes. Late anaphase. Beginning formation of first polar body. Deep aster with large clear centrosphere. Dyads have reached margin of centrosphere.

FIG. O. 20 minutes. Telophase. Extrusion of first polar body. Mid-body and spindle remnant. Deep centrosphere cut obliquely. Dyads have moved a little apart, forming irregular circle at boundary of centrosphere.

FIG. P. 35 minutes. Spindle remnant has disappeared. Beginning disintegration of aster. Spherical centrosphere, capped by dyads. Early sperm aster (lower right) in neighbourhood of maturation aster.

PLATE 2

FIGS. A–M. Formation of second maturation spindle and completion of second maturation division in *Limnaea stagnalis.*

FIG. A. 30 minutes. Elongation of centrosphere. Centriole in outer 'focal point' of elliptical centrosphere. Dyads arranged in loose circle about outer pole of centrosphere.

FIG. B. 25 minutes. Centrosphere becomes egg-shaped. Centriole has nearly reached its pointed outer pole. Beginning of longitudinal striation in outer half of centrosphere. Dyads have moved along surface of centrosphere towards equatorial region.

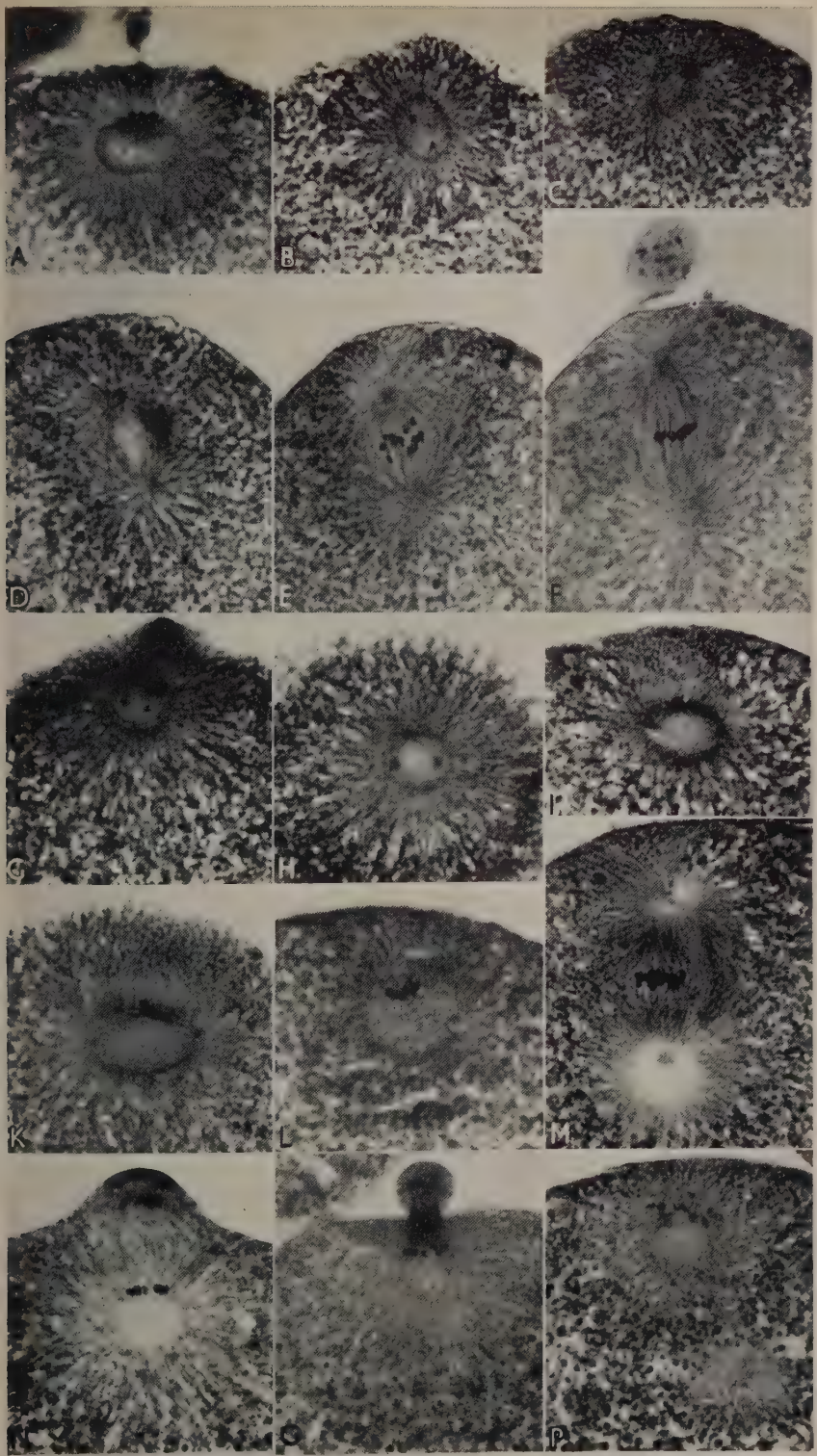
FIG. C. 40 minutes. Egg-shaped centrosphere. Centriole has reached outer pole, beginning formation of aster. Deep end of centrosphere blunt without centre or aster. Longitudinal striation in outer half of centrosphere. Dyads have further moved on towards equator.

FIG. D. 50 minutes. Sperm aster approaches inner end of egg-shaped centrosphere (the latter is cut obliquely). Astral rays of sperm aster on side of centrosphere shorter than on other sides.

FIG. E. 40 minutes. Sperm aster has fused with inner end of centrosphere (the latter is cut obliquely). Layer of small granules at boundary between the two.

FIG. F. 45 minutes. Early second maturation spindle. Small outer aster without centrosphere, large inner aster with big clear centrosphere. Layer of granules and small vacuoles at boundary between spindle and centrosphere. Dyads still forming irregular circle outside spindle in supra-equatorial region.

FIG. G. 45 minutes. Early second maturation spindle. Asters as in previous figure. Layer of granules and vacuoles distinctly visible. Dyads begin to penetrate into spindle.



CHR. P. RAVEN, F. C. M. ESCHER, W. M. HERREBOUT, *and* J. A. LEUSSINK

Plate 1

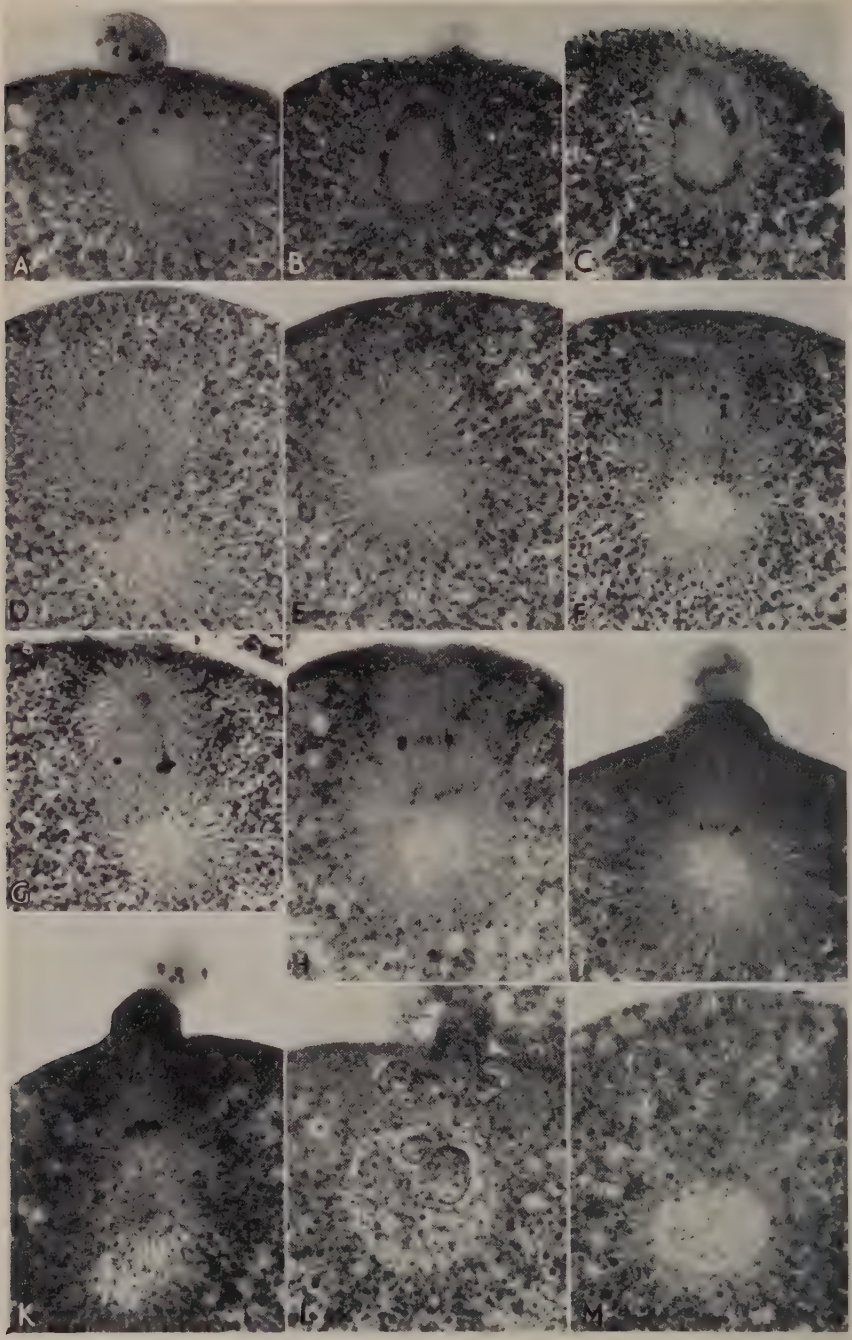

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FIG. H. 85 minutes. Metaphase of second maturation division. Deep aster with big, clear centrosphere; superficial aster without centrosphere begins to flatten against surface. Small indentation of egg surface. Zone of granules at boundary between spindle and deep centrosphere still present. Dyads arranged in equatorial plane.

FIG. J. 80 minutes. Late anaphase. Beginning formation of second polar body. Dyads have reached margin of deep centrosphere.

FIG. K. 95 minutes. Late anaphase to telophase. Formation of second polar body. Deep aster begins to move into depth.

FIG. L. 120 minutes. Egg chromosomes have formed karyomeres beneath egg cortex at animal pole. Sperm nucleus has migrated to animal pole, lies beneath egg karyomeres in disintegrating sperm aster.

FIG. M. 90 minutes. Sperm aster, after having freed itself from remnant of maturation spindle, has moved into depth. Strong vacuolization of centrosphere, disintegrating fringe of astral rays.

(Manuscript received 8 : iv : 57)

The Localization of β-Glucuronidase in the Early Chick Embryo

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WITH ONE PLATE

INTRODUCTION

In vertebrate tissues there appears to be a connexion between β-glucuronidase and the proliferation of cells. This connexion was first noticed by Levvy, Kerr, & Campbell (1948) when they were investigating the effect of toxic compounds on mouse-liver glucuronidase. The early stages of embryonic development are characterized by rapid proliferations of cells and it is, therefore, of some interest to study the localization of β-glucuronidase during these stages. Small amounts of β-glucuronidase can be detected in the early embryos of *Xenopus laevis* (Billett, 1956) and in those of *Drosophila melanogaster* (Billett & Counce, unpublished). In these embryos no marked increase in the enzyme can be associated with the proliferation of cells. The large amount of yolk in the *Xenopus* and *Drosophila* embryos was a complicating factor in the above experiments. It was not possible to localize the enzyme in these embryos with a histochemical method. An embedding technique suited to the nature of the embryos and the requirements of the histochemical test could not be devised.

It has, however, proved relatively simple to apply a modification of the Friedenwald & Becker (1948) technique to whole chick embryos cultivated *in vitro*. This technique, because of the diffuse nature of the reaction and the destruction of cytological detail which it entails, can only demonstrate the presence of β-glucuronidase in particular cells or groups of cells (Billett & McGee-Russell, 1956). Intracellular localization of the enzyme is impossible. The method appears to be well suited to the present purpose, aimed at a histological rather than a cytological localization of the enzyme in the early chick embryo.

METHODS

The modification of the Friedenwald & Becker technique, used in these experiments, has been described elsewhere (Billett & McGee-Russell, 1955).

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Chick embryos, which varied in age from the primitive streak to 10 or more somites, were transferred to culture chambers according to the method described by New (1955). About an hour after explantation the albumen, which is in contact with the dorsal side of the embryos, was removed and the embryos were completely surrounded by a saturated solution of ferric 8-hydroxyquinoline and quinolyl-8-glucuronide in 0·1 M acetate buffer at pH 4·5. The embryos were incubated in the substrate mixture for 24 hours at 37° C. Development was arrested by the substrate, and at the end of incubation the embryonic tissues appeared to have suffered little distortion. After this treatment the embryos were removed from the culture chambers, washed in water, and the vitelline membranes removed. The embryos were then washed in oxalate buffer, again in water, and then placed for half an hour in neutral formaldehyde solution (4 per cent. w/v). After a final washing in water the embryos were mounted in Farrant's medium. The formation of brown crystals of ferric 8-hydroxyquinoline, embedded in the tissue, was taken to indicate the presence of the enzyme.

Controls were set up in three ways: (1) substrate mixture was prepared without the addition of glucuronide; (2) embryos were heated to 80–90° C. for 5 minutes; (3) substrate was used containing 0·001 M potassium hydrogen saccharate. The latter is a specific inhibitor for the enzyme (Levvy, 1952).

RESULTS

Our findings are summarized in Table 1. Typical preparations are shown in the Plate. The enzyme could not be detected in the primitive streak stage. In 5

TABLE 1

Stage no. of Hamburger & Hamilton (1951)	*Description of stage*	*Glucuronidase reaction No. of embryos*		
		Strongly positive	*Weak reaction*	*No reaction*
3–4	Primitive streak	0	1	8
5	Head-process	5	0	4
6	Head-fold	1	0	9
7–8	1–3 pairs somites	8	2	3
8–9	4–6 pairs somites	7	0	0
9–12	9–12 pairs somites	6	0	2

out of 9 cases the head-process stage gave a strongly positive reaction. At a slightly later stage, formation of the head-fold, only 1 positive reaction out of 10 was recorded. These observations suggest that the enzyme disappears at this stage. Alternatively, a technical failure of the histochemical reaction would account for the result. However, the results for the head-fold embryos were obtained from four different batches of embryos which contained earlier and

later stages which gave positive results. Once the somites had formed, a strong positive reaction was usually obtained.

An examination of the preparations gave a general impression that the enzyme was at first widely diffused on the dorsal side of the embryo, and that it later became more strictly localized in the mid-dorsal line in the region of the neural tube and somites. Little or no enzyme could be detected on the ventral side of the embryo. Crystals were only deposited in the embryos and in the extra-embryonic tissue immediately surrounding the embryo. At the head-process stage crystals were deposited in the region of the primitive streak. In the later stages crystals were rarely deposited in the retreating streak, in marked contrast with the remainder of the embryo (Plate, figs. B–D).

Some of the treated embryos were embedded in 10 per cent. w/v gelatin and sectioned. Satisfactory embedding was not achieved because the crystals of ferric 8-hydroxyquinoline dissolved if left in the aqueous gelatin at 37° C. for more than 6 hours. Only a short embedding time, of approximately 2 hours, could be used, and this made sections difficult to cut. Examination of the sections showed crystals of ferric 8-hydroxyquinoline embedded in the neural folds and adjacent ectoderm, and in the somites.

The effect of potassium hydrogen saccharate on the development of the embryos

Although a 0·001 M solution of potassium hydrogen saccharate prevented the formation of ferric 8-hydroxyquinoline in the histochemical test, a similar concentration of the inhibitor did not prevent the normal development of embryos explanted at a primitive streak stage. This observation suggests that the enzyme is either inactive or inaccessible in the intact embryo. In this connexion it is to be noted that Karunairatnum & Levvy (1949) failed to influence liver regeneration and growth in mice by the administration of large doses of saccharic acid.

DISCUSSION

Using the method described above, β-glucurinodase cannot be detected in chick embryos until the tissues have begun to differentiate. These results do not support a general hypothesis that the enzyme is specifically associated with the proliferation of cells.

Hollinger & Rossiter (1952), studying Wallerian degeneration of nerve, observed that an increase in β-glucuronidase occurred after cellular proliferation had taken place in the degenerating tissue. In the liver of the rat, regenerating after sub-total hepatectomy, the peak of β-glucuronidase activity also occurs after the phase of rapid cellular proliferation (Mills *et al.*, 1950).

These results for regenerating tissue, and the present findings for the chick embryo, suggest that β-glucuronidase is often associated with tissues which are in the process of differentiation following an initial phase of cellular proliferation. Changes in β-glucuronidase activity may in fact reflect changes in cell-

type, as was suggested by Mills & Smith (1951). Raised β-glucuronidase levels, such as occur in certain neoplasms (Fishman *et al.*, 1947, 1950) may indicate a type of tissue whose cells have reverted to, and remain in, a state which is characteristic of those found in tissues in the early stages of differentiation.

SUMMARY

The technique for the localization of β-glucuronidase, based on the precipitation of ferric 8-hydroxyquinoline from quinolyl-8-glucuronide has been applied to explanted chick embryos. When the enzyme is first detectable, at the head-process stage, it appears to be widely diffused in the dorsal side of the embryo and in the adjacent area opaca. In later stages the histochemical reaction is confined to the region of the developing somites and neural tube.

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EXPLANATION OF PLATE

The photographs are of whole chick embryos seen from the dorsal side. The crystals of ferric 8-hydroxyquinoline appear as black spots. The preparations are unstained.

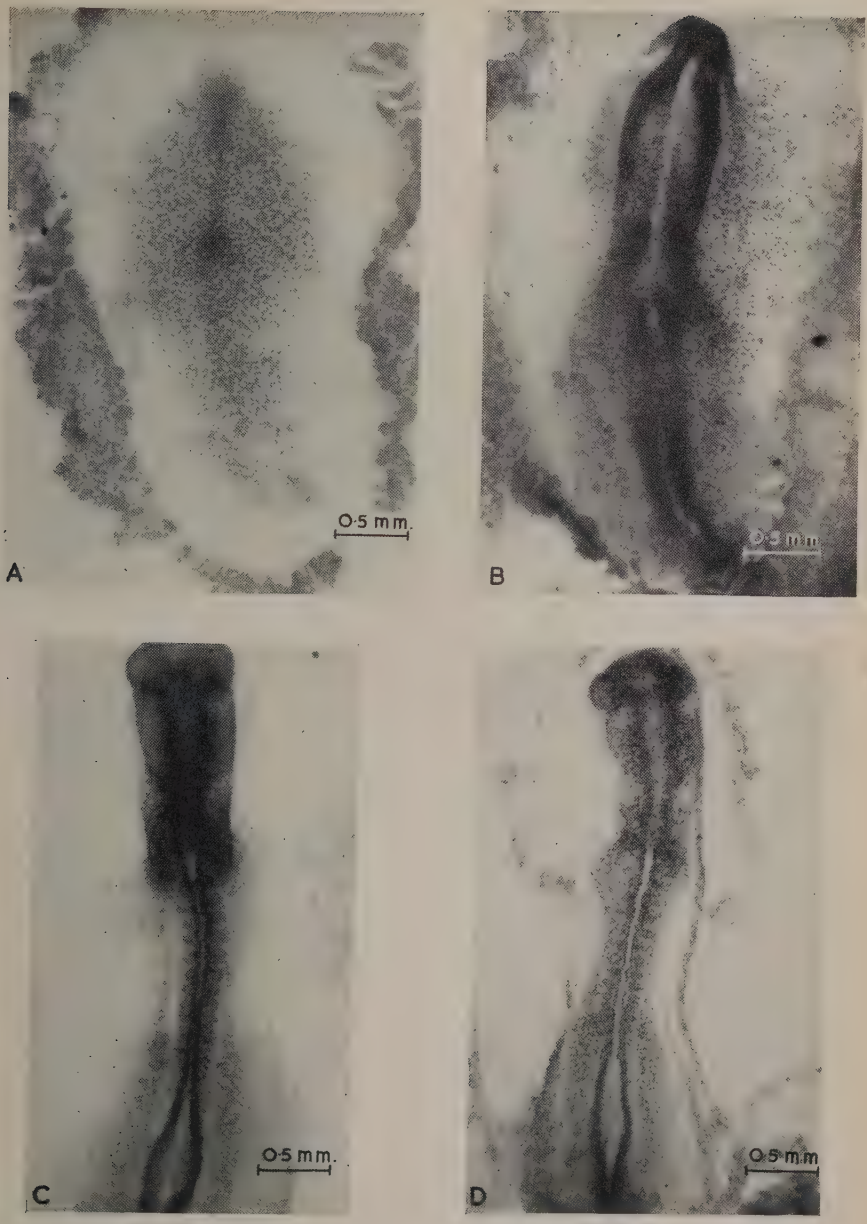
FIG. A. Head-process. Test. Deposits of ferric 8-hydroxyquinoline in the dorsal side of embryo and in the adjacent area pellucida.

FIG. B. Three pairs of somites. Test. Deposits of ferric 8-hydroxyquinoline in dorsal side of embryo and in area pellucida on each side of the embryo.

FIG. C. About ten pairs of somites. Test. Deposits of ferric 8-hydroxyquinoline concentrated in mid-dorsal line, in neural tube and somites.

FIG. D. About twelve pairs of somites. Similar to fig. C.

(Manuscript received 9 : iv : 57)



F. BILLETT *and* L. MULHERKAR

Observations on Rabbit Blastocysts Prepared as Flat Mounts

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WITH ONE PLATE

THE structure of the mammalian blastocyst has generally been studied on whole mounts in fluid, or on sections of embedded preparations. If the blastocyst of the rabbit is split open and mounted flat, however, the intact cells in their correct topographical relationships may be examined under the high powers of the microscope. This technique, which is quick and simple, may be employed in cytological and cytochemical studies of the large blastocyst cells, in study of mitotic patterns in the blastocyst wall, and in evaluation of the effects of culture media and of various experimental techniques applied to the blastocysts themselves or to the mother.

MATERIAL AND METHODS

The pregnant animals (6–6½ days' post-coitum) were handled according to the methods previously described (Lutwak-Mann, 1954). In most cases the doe was anaesthetized by intravenous injection of nembutal, and the uterus removed and slit open. The blastocysts were picked out with a small stainless steel spoon and then quickly rinsed in physiological saline and fixed in absolute methanol for 1 hour or longer. The saline rinse is essential, since if it is omitted the acellular outer membrane curls after the vesicle is torn and thus prevents mounting; apparently some substance that shrinks strongly on drying is rinsed off. Ethanol, Bouin's fluid, and Mayer's picro-nitric fixative with added osmic acid, which Hartmann (1916) used for opossum blastocysts, all caused crumpling of the trophoblast. Even in methanol the trophoblast occasionally crumpled, rendering the preparation useless.

In mounting, one fixed blastocyst is placed with the embryonic shield downwards on a cover-slip in a flat dish containing just enough methanol to submerge the vesicle. The abembryonic pole is punctured with fine steel needles, and the

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trophoblast, together with the covering zona pellucida, is torn into strips by slits that are carried to the edge of the shield. The fluid is drained off, and the star-shaped preparation allowed to dry on its cover-slip. Though a certain amount of wrinkling is inevitable, expanses of smoothly flattened trophoblast and adherent endoderm are nevertheless obtained (Plate, fig. 1). Generally the entire blastocyst is mounted, but, if desired, as, for example, for a series of histochemical tests, a single blastocyst may be readily torn into 5 or 6 longitudinal strips which are each mounted separately. Seven-day blastocysts may also be prepared as flat mounts, but the technique is somewhat more difficult because the trophoblast, having a tendency to separate from the zona pellucida at this stage, is quite fragile.

For staining, the cover slip bearing the blastocyst is dropped into the stain solution. Most often we have used Mayer's acid hemalum, but Delafield's hematoxylin is also satisfactory if it is acidified to minimize staining of the zona pellucida. In either case, the stain is applied for 20–40 minutes, after which the preparation is washed with tap-water. Other stains which we used were the Feulgen (6 minutes' hydrolysis in *N* HCl at 60° C., followed by 1 hour in the Schiff reagent) and the Unna-Pappenheim or 0·2 per cent. pyronine alone (both prepared in a pH 4·8 acetate buffer and applied for 2 hours). None of these nucleic acid stains coloured the zona pellucida. After staining, the blastocysts were dehydrated and mounted in a neutral medium.

THE NORMAL BLASTOCYST

In almost all our preparations the trophoblast and endoderm are underlain by zona pellucida and associated covering materials (cf. Boving, 1954). Staining of these non-cellular layers could not be entirely suppressed when hematoxylin is used, and consequently the whole preparation generally took on a pale purplish hue. Usually it was not possible to distinguish zona directly from trophoblast, but sometimes the two layers slipped apart at the torn edges, and it was then possible to see that the cytoplasm, particularly in the abembryonic region, is tenuous and almost uncoloured. The zona, on the other hand, appeared equally, though lightly, stained from the shield to the opposite pole. The shield cells themselves stained intensely, and an abrupt transition may be seen from these small dark cells to the large pale cells of the extra-embryonic area. The shield itself was in most cases featureless at the stage we used, but occasionally showed the first signs of primitive streak formation. In the abembryonic region, to which the endoderm has not extended at $6\frac{1}{2}$ days, cell-walls sometimes stand out (Plate, fig. 2), but they are not otherwise seen. Within any given area the cells appear to be of approximately equal size, but irregularly we noted in the abembryonic hemisphere small cells arranged in clusters which might be the precursors of the trophoblastic knobs (Schoenfeld, 1903). In the endoderm the nuclei are smaller and darker, thus appearing more condensed, than those of the trophoderm (Plate, fig. 3). During prophase, however, the

endodermal nuclei seem to expand, so that it is not possible to distinguish endodermal from trophoblastic mitoses in the area where both layers occur.

When the Unna-Pappenheim procedure is used the cytoplasm in the embryonic hemisphere is found to be rich in brilliant red granules. Such granules are also present in the abembryonic area in the previously mentioned clustered small cells, but since the granules are otherwise completely lacking from trophoblast that has not been underlain by endoderm, they probably are present in the endoderm. The resting nuclei are very pale. When pyronin is used alone the nucleoli of the trophoblastic cells stain, whereas in the endoderm the entire nucleus is reddened. Since the tendency to stain with pyronin is abolished from both nuclear and cytoplasmic sites by extraction with cold 10 per cent. perchloric acid, it appears that ribonucleic acid is present in all red-staining sites. After Feulgen treatment all resting nuclei present a fine purple-stained network with usually a single mass (probably a sex chromosome) lying against the nuclear membrane.

RATE AND DISTRIBUTION OF MITOTIC ACTIVITY

Mitotic figures are easy to recognize in the large, intact nuclei of the blastocyst wall and may be readily counted at 440 × magnification. For counting mitoses we used a line cut in a thin glass plate inserted in the microscope ocular. One 'arm' of a preparation was chosen, and a narrow strip of it at the abembryonic end moved across the field with all cells transected by the line at each random turn of the mechanical stage screw being counted; in effect this is a simple adaptation of the method of Chalkley (1943) for measuring a cellular component. After one strip was counted a neighbouring strip was brought into

TABLE 1

Mitotic activity in different areas of the blastocyst wall. (For description of areas, see text)

Area	*No. of cells counted*	*No. of cells in mitosis*	*Percentage of cells in mitosis*
Abembryonic trophoblast	5,444	644	11·8
Outer endoderm+trophoblast	8 550	982	11·5
Inner endoderm+trophoblast	7,422	798	10·7
TOTAL	21,416	2,424	11·2

place and the process repeated until the entire 'arm' from abembryonic pole to shield margin had been examined. The data were recorded for trophoblast only (roughly the abembryonic third of the system), the outer endoderm plus overlying trophoderm (equatorial third), and the inner endoderm plus overlying trophoderm (the remainder, not including the shield). Counts of 12 'arms' in 6 preparations from 3 litters gave the results shown in Table 1. There is no

significant difference in proportion of cells that are dividing at a given time in different parts of the system.

When the phases of mitosis are considered separately (Table 2), a deficiency in percentage of anaphases in the pure trophoderm suggested a possible difference in rate for this phase between endoderm and trophoderm, or perhaps

TABLE 2

Percentage of mitotic figures in the phases of mitosis in different areas of the blastocyst wall

Area	*Percentage of dividing cells in*			
	Prophase	*Metaphase*	*Anaphase*	*Telophase*
Abembryonic trophoblast	55·7	19·5	8·4	16·4
Outer endoderm+trophoblast	55·3	18·5	11·4	14·8
Inner endoderm+trophoblast	58·4	20·3	11·3	10·0
TOTAL	56·3	19·4	10·6	13·7

TABLE 3

Relative proportions of metaphases and anaphases in the embryonic and abembryonic hemispheres of the blastocyst wall

Hemisphere	*No. of metaphases*	*No. of anaphases*	*M/A*	*P*
Embryonic	978	697	1·40±0·11*	0·01
Abembryonic	551	212	2·59±0·24	

* Standard error of the mean.

between embryonic and abembryonic trophoderm. To investigate this possibility further, without the labour of complete mitotic counts, we adopted the practice of counting all metaphases and anaphases, but not other stages, in single 'arms'. This procedure, which the large size of the blastocyst cells makes very facile, is justified by the relatively constant proportions of metaphases in different regions. When these results were scored for the three areas, the ratio of metaphases to anaphases was significantly higher in the abembryonic third than in the embryonic (mean difference, 0·85; standard error of mean, 0·05; $P < 0{\cdot}01$). The ratio in the equatorial third was intermediate between the other two, but, because of the greater variability in the equatorial strip, the difference between this and either of the others was not significant. A striking difference is, however, obtained if the results are scored by hemispheres, as Table 3 shows. A similar result is also obtained if the data are compared as M/A ratios for the two halves of single 'arms', instead of being pooled. Since the abembryonic half or third is predominantly or solely trophoblastic, these results apparently indicate that the

time spent in anaphase is relatively less in trophoblast than in endoderm. The results may also indicate a gradient in anaphase rate in one or both layers, but the similar appearance of endodermal and trophoblastic nuclei in mitosis makes this possibility difficult to evaluate.

EXPERIMENTAL OBSERVATIONS

It has long been known that the fully expanded free blastocyst is not amenable to maintenance in culture (Pincus, 1936). The technique described in this paper offers a simple means of assaying the effects of various treatments and media, for we have observed that the nuclei, particularly in the abembryonic area, are swiftly affected by exposure to extra-uterine agencies. A slight deficiency in number of anaphases, indicating metaphase arrest, is the first sign of impairment; in many preparations, the metaphase figures appeared perfectly normal, even when the anaphases were greatly reduced in number. A more severe effect is signalized by clumping of the metaphase chromatin, and the most seriously damaged blastocysts also show marked nuclear abnormality, including condensation of chromatin and appearance of vacuoles in and around the nuclei (Plate, fig. 4). The effects are always most striking in the abembryonic hemisphere, and sometimes only a small area around the pole is affected. Under the treatments we have used, nuclear condensation and vacuolization were rarely seen in the embryonic hemisphere.

1. *Influence of medium*

Routinely we tested the effect of exposing blastocysts to culture media for 20 minutes at 37° C. before fixing; 2 ml. of medium were used for each 4–6 blastocysts. Representative results, expressed as M/A ratios for entire 'arms', are given in Table 4. These results, and others we have obtained, suggest that Tyrode solution is a relative favourable medium for short-term maintenance; but a different conclusion emerges if we examine the effect on the two hemispheres separately of the litter D90 blastocysts (Table 5, part A). The sensitive abembryonic hemisphere clearly shows mitotic arrest. Moreover, not all litters gave such a favourable response to Tyrode as did D90. Some suffered mild or strong mitotic inhibition in both hemispheres, as is shown in the pooled data from a large number of counts (Table 5, part B). The variation in response is to be attributed to litter more than to individual differences, for we found that, with occasional exceptions, the members of a single litter tend to be affected uniformly by a given treatment. For this reason it is essential to have some littermate controls in Tyrode (or other medium) alone, whenever the effect of any chemical substance added to (or substracted from) the medium is tested.

Fluid aspirated from blastocysts seemed to alleviate the deleterious effects of Ringer-phosphate (Table 4, litter C76). When used in combination with Tyrode, 8-day fluid gave us the most favourable results we ever obtained, for the blasto-

cysts kept in this solution showed only a slight increase in M/A ratio, even in the abembryonic region (Table 4; Table 5, part C), with no sign of metaphase clumping or other nuclear damage. Clotting of the 8-day fluid around the test

TABLE 4

Relative proportions of metaphases and anaphases in blastocysts exposed to various media for 20 minutes. Controls were litter-mates fixed immediately after rinsing

Litter	*Medium*	*No. blastocysts*	*No. metaphases*	*No. anaphases*	*M/A*
E 59	Control	3	206	153	1·53
	Ringer-bicarbonate*	3	255	56	4·55
D 90	Control	4	417	197	2·12
	Tyrode	4	567	225	2·52
C 76	Ringer-phosphate*	3	184	5	36·8
	Ringer-phosphate+rabbit serum (3:1)	2	165	0	
	Ringer-phosphate+8-day blastocyst fluid (3:1)	3	354	137	2·58
	Ringer-phosphate+7-day	1	123	26	4·70
D 73	Control	3	239	129	1·85
	Tyrode+8-day blastocyst fluid (3:1)	4	435	179	2·43

* Blastocysts in this group showed incipient pycnosis.

TABLE 5

Relative proportions of metaphases and anaphases in the embryonic and abembryonic hemispheres of blastocysts maintained in Tyrode or Tyrode with 8-day blastocyst fluid for 20 minutes. Controls as in Table 4

Treatment	*No. of litters*	*No. blastocysts*	*Embryonic hemisphere*			*Abembryonic hemisphere*		
			M	*A*	*M/A*	*M*	*A*	*M/A*
A. Control	1 (D 90)	4	265	149	1·78	152	52	2·92
Tyrode	1 „	4	347	209	1·66	214	16	13·4
B. Control	4	11	768	486	1·21	399	161	2·45
Tyrode	9	30	2,259	905	2·54	1,520	263	5·77
C. Control	1 (D 73)	3	151	87	1·72	88	42	2·09
Tyrode+8-day blastocyst fluid (3:1)	1 „	4	280	117	2·39	155	62	2·50

blastocysts made them difficult to handle, but may have contributed to their good survival. An experiment in which five blastocysts were kept in Tyrode with 7-day fluid unfortunately gave equivocal results, for the controls maintained in Tyrode alone had an abnormally low proportion of anaphases; the experimentals showed no improvement as compared with their controls.

Although we made no detailed counts of mitoses in the embryonic shield, we nevertheless noted that the mitotic pattern appeared normal even when the abembryonic trophoblast showed severe damage. This finding is not unexpected, since the shield will develop to a limited extent in culture when separated from the rest of the system (Waddington & Waterman, 1933).

2. *Age*

Comparison of treated 6- and 6½-day blastocysts indicated no difference in ability to survive 20 minutes' exposure to Tyrode or Ringer-bicarbonate solution.

3. *Temperature, anaerobiosis, and anaesthesia*

It was thought that temperature shock or oxygen lack or the nembutal administered to the mother might be responsible for the inability of the blastocysts to continue normal mitotic activity in culture. The first possibility was eliminated by placing the freshly excised uterus in a chamber maintained at 37° C. in which all subsequent operations up to fixation were carried out. Exposure of blastocysts so handled to Tyrode solution gave results that were not better than in experiments in which the blastocysts were removed at room temperature. To check on the possibility that oxygen lack has an inhibitory effect, we left one uterine horn with its contained blastocysts intact for 30 minutes after the death of the mother, while the blastocysts of the other horn were removed as quickly as possible, rinsed, and fixed at once. The preparations of the two groups (four in each group) could not be distinguished from each other by their appearance. There was no significant difference, either in the embryonic or the abembryonic hemispheres, between their M/A ratios; and prophases, which are most sensitive to oxygen deprivation (Bullough & Johnson, 1951), were abundant in both groups. The effect of nembutal was examined by administration to a pregnant animal of 1·5 ml. of a solution containing 60 mg. of the anaesthetic per kilogramme. The treated doe was killed by a blow on the head 30 minutes later. Six blastocysts were then removed and fixed after brief rinsing. They did not differ, either in general appearance or in M/A ratio, from those of litters removed promptly after nembutal injection.

4. *Superovulation*

Because of the desirability of having adequate numbers of controls within a single litter, we tested the effect of superovulation induced by a gonadotrophin preparation according to the procedure of Chang (1948). Of two does thus treated we obtained 41 blastocysts from one and 46 from the other. The first group were definitely ellipsoidal and apparently near implantation; they are difficult to spread at this stage and were discarded. Of the larger batch, eight were selected at random and mounted. Of these, one, which stained only lightly, showed no mitotic figures, but the others were indistinguishable from blastocysts of normal- sized litters.

5. *Stilboestrol*

Since stilboestrol, like other oestrogens, is effective in preventing nidation, we looked into the possibility that this substance acts directly on the blastocysts. Five days after mating, two young does were given 5 mg. of stilboestrol by subcutaneous injection; 32 hours later they were sacrificed and their blastocysts (2 from one, 6 from the other) were fixed after rinsing. One blastocyst of the larger litter had almost no cells in mitosis, but the other seven appeared quite normal.

6. *Aminopterin and citrovorum factor (leucovorin)*

The folic acid antagonist aminopterin arrests mitosis in metaphase within 15 minutes when applied at concentrations as low as 1:20,000 to chick embryo tissues in culture (Jacobson, 1954). We maintained blastocysts for 20 minutes in Tyrode solution containing from 0·025 to 0·2 mg. of aminopterin per ml.; there was no sign of metaphase abnormality, and the M/A ratios did not differ significantly from those of litter-mates maintained in Tyrode only. In one experiment in which the aminopterin concentration was raised to 0·5 mg. per ml., there was an almost complete absence of anaphase figures from the five treated specimens; but the controls of this litter themselves showed signs of metaphase inhibition, with abnormally high M/A ratios. The failure of aminopterin to exert any effect suggested that the zona pellucida might have inactivated the inhibitor or prevented its passage into the blastocyst itself. To check this point we tried slitting the vesicles open before culturing them in order to permit the aminopterin to reach the inside of the system; but this treatment invariably resulted in crumpling of the trophoderm, which could not subsequently be spread. Citrovorum factor (leucovorin), used at 0·25 mg. per ml., had no clear-cut effect, either alone or in combination with aminopterin.

DISCUSSION

The fully expanded rabbit blastocyst, at the time when it is almost ready to be implanted, has long been known to be in a critically sensitive condition, whether *in utero* or in culture. Beyond this stage, the embryonic disk, at least, develops readily *in vitro*. Earlier, morulae and young blastulae can be raised in aerated serum up to the condition of the 6-day blastocyst (Pincus & Werthessen, 1938). When the blastocyst of 5–6 days after coitus is placed in culture, however, it generally collapses in a relatively short time (Brachet, 1913; Waterman, 1933), although Chang (1950) has shown that 6-day blastocysts isolated for as long as 45 minutes may develop normally if transplanted to a pseudopregnant uterus. That the behaviour of the blastocyst in the sensitive period is under external control has been accepted since Corner (1928) showed that removal of the ovaries or corpora lutea shortly after mating terminates the pregnancy at the blastocyst stage. Although it is clear that progesterone plays an essential role,

the nature of the uterine conditions that carry the blastocyst through the critical period is not yet understood.

The findings we have presented in this paper suggest that internal factors are also of importance, for the mitotic mechanisms of the 6-day blastocyst seem to be extraordinarily susceptible to the influence of external agents. This susceptibility is not a general property of the rabbit egg, for cleavages go on readily in culture (cf. Pincus, 1936). It might be thought that the sensitivity of the blastocyst nuclei is merely a secondary result of the thinning of the external coverings, particularly in the abembryonic region, prior to implantation. If this were the correct explanation, however, one would expect 6-day blastocysts to be more resistant to mitotic inhibition than those of $6\frac{1}{2}$ days, but this is not so. Moreover, although we had no means of estimating the exact thickness of the covering coats, our flat mounts do in all cases show a firm, dense zona pellucida underlying the delicate trophoblast in abembryonic as well as embryonic areas. Boving (1954) has shown in addition that Tyrode solution does not tend to dissolve the gloiolemma.

Whether the mitotic suppression that we have observed in culture is due to the presence of an inhibitor, or lack of an essential nutrient, cannot be decided from our data; both effects may be involved. The severe inhibition caused by Ringer's solution with phosphate is interesting in light of the fact that at this stage the blastocyst fluid contains very little phosphate (Lutwak-Mann, 1954). The protective influence exerted against the phosphate medium by 8-day blastocyst fluid should be examined further. Possibly the fluid binds the phosphate; or it might contain some substance required for the mitotic process. The clotting of the 8-day fluid does not seem to be the critical factor, for the blastocysts remain firmly distended, and carry on mitotic activity, in fluid that offers no special mechanical support. On the other hand, the clotting might well be a useful adjunct to continued culture, for the best survival of isolated blastocysts has involved embedding them in plasm clots (Brachet, 1913; Waterman, 1933).

Our results as yet do not shed much light on the biochemical mechanisms underlying mitosis in the blastocyst. The ineffectiveness of oxygen deprivation is not surprising, for pre-implantation blastocysts are probably adapted, as are many other organisms in early developmental stages (cf. Bullough, 1952), to very low oxygen tensions. The ineffectiveness of aminopterin might be indicative of some fundamental difference between the mitotic process of the blastocyst and those of many older tissues (Jacobson, 1954); but this matter remains equivocal because the inhibitor may have failed to penetrate the outer coats of the egg, or may have been inactivated by them. The failure of a large dose of stilboestrol to injure the blastocysts or impair their mitotic activity is another indication that oestrogens interfere with implantation in an indirect way (Pincus & Kirsch, 1936).

The greater sensitivity to mitotic inhibition of the abembryonic hemisphere as compared with the embryonic might be an artefact, resulting from greater

permeability in the abembryonic region, but is probably a real phenomenon. The fact that the zona pellucida and associated coverings are not obviously thinned toward the abembryonic pole has already been pointed out. Boving (1954), moreover, has demonstrated a distinct chemical difference between the two hemispheres. Such a difference is reflected in our observations both in the different mitotic rate of the abembryonic trophoblast and also in the weak hematoxylin staining of the nuclei and particularly of the nucleoli of this material. The fact that it is always the pole opposite to the embryonic shield that implants also indicates that the vesicle is chemically non-uniform. The differential sensitivity of the dividing nuclei of different areas of the blastocyst may well be an aspect of this non-uniformity.

SUMMARY

1. A technique is presented for splitting fixed blastocysts open and mounting the entire vesicle on a flat surface. This technique permits close examination of all the cells of the vesicle in a single preparation. The appearance of the extra-embryonic cells when stained with hematoxylin or by nucleic acid techniques is described. Superovulated blastocysts showed no evident differences from those of normal-sized litters.

2. The extra-embryonic layers have a mitotic rate of 11·2 per cent., which appears to be uniform in the endoderm and different areas of the trophoblast. The abembryonic trophoblast, however, seems to be relatively deficient in number of anaphases, and the difference in the ratio of metaphases to anaphases in the embryonic and abembryonic regions is highly significant. Apparently anaphases are completed more quickly in the abembryonic trophoblast than elsewhere.

3. Exposure of blastocysts to various culture media for 20 minutes before fixing frequently resulted in damage to the nuclei in both resting and mitotic states. The abembryonic nuclei are especially sensitive, and metaphase arrest regularly occurs to a greater extent in this hemisphere than in the embryonic. Nuclear integrity appeared to be sustained best in media containing fluid aspirated from 8-day blastocysts.

4. Blastocysts of 6 and $6\frac{1}{2}$ days did not differ in ability to survive 20 minutes' maintenance in fluid culture. Temperature shock, anaerobiosis, and maternal anaesthesia were also ruled out as causal factors in the nuclear damage occurring during culture. Administration of a large dose of stilboestrol to the pregnant doe did not result in any apparent injury to the blastocysts.

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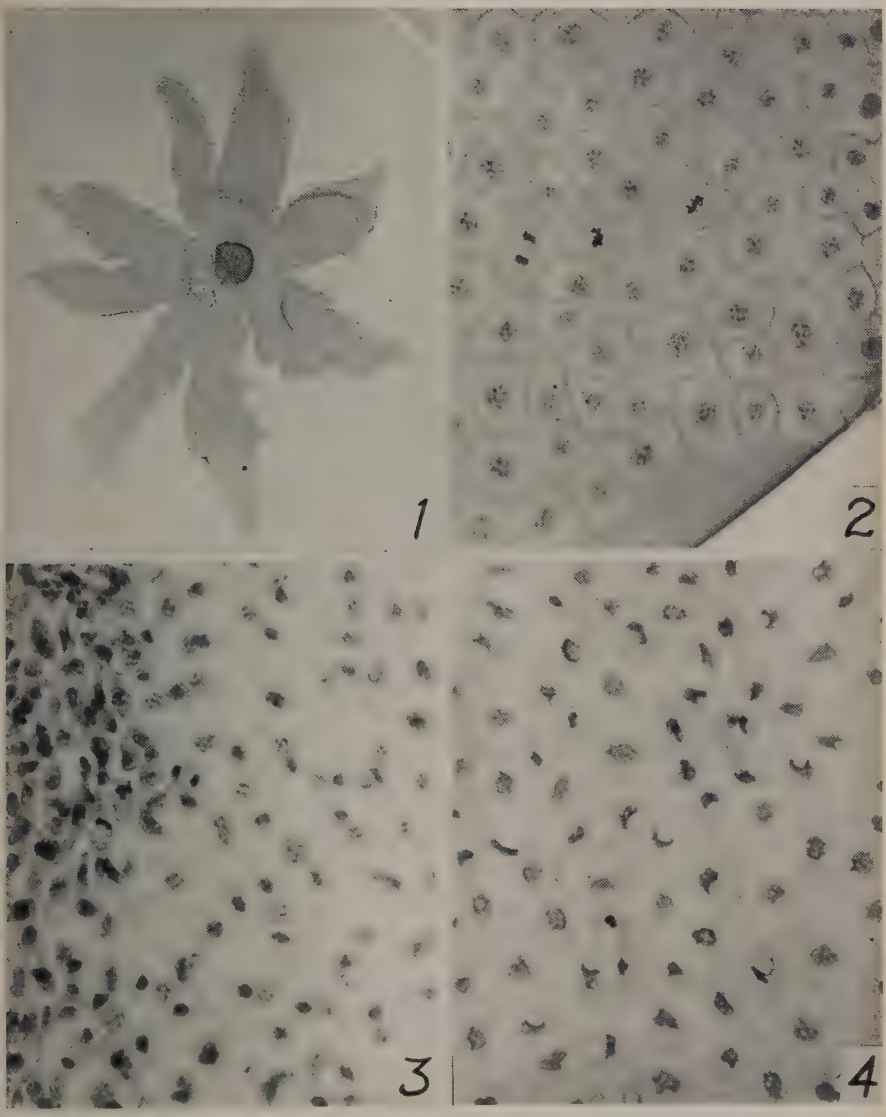

F. MOOG *and* C. LUTWAK-MANN

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EXPLANATION OF PLATE

All preparations shown in this plate were stained with Mayer's acid hemalum. Fig. 1 was photographed at a magnification of 6×, the others at 250×.

Fig. 1. An entire 6½-day blastocyst spread on a cover-slip. The dark-stained mass at the centre is the embryonic shield.

Fig. 2. Area of abembryonic trophoblast showing cell-walls. In some preparations the cell boundaries are indistinct or cannot be seen at all. Along the cut edge (lower right) there is an area of zona pellucida not covered by trophoblast.

Fig. 3. Area of embryonic trophoblast and endoderm. The edge of the embryonic shield may be seen in the upper left corner. In the extra-embryonic area the endodermal and trophoblastic nuclei may be distinguished by the fact that the former are smaller, darker, and generally have fewer nucleoli than the latter.

Fig. 4. Area of abembryonic trophoblast showing clumping of chromatin and vacuolization of nuclei and cytoplasm. This kind of damage frequently occurs when the blastocysts are exposed to culture media.

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The Early Development of the Interorbital Septum and the Fate of the Anterior Orbital Cartilages in Birds

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WITH ONE PLATE

INTRODUCTION

In most reptiles and almost all birds there is a thin vertical sheet of skeletal material between the orbits known as the interorbital septum which is continuous in front with the nasal septum. In reptiles the interorbital septum remains cartilaginous and there are usually fenestrae in it filled with membrane; in birds it often becomes to a large extent ossified although fenestrae may still occur.

There is evidence that in the embryo the interorbital septum is derived, partly at least, from the fusion of two pairs of cartilaginous or procartilaginous primordia; the preoptic roots of the orbital cartilages above, and the trabeculae below (see Bellairs, 1949). It would also seem, however, that in some forms at least, the interorbital septum has a third component which develops in the midline between the trabeculae and merges with them and the orbital cartilages to form the definitive interorbital septum. This third component was originally called the intertrabecula by W. K. Parker (1880) in his study of the development of the turtle.

Among the birds an intertrabecula in the chick has been described by Parker (1891) and in the kestrel by Suschkin (1899). More recent workers on the chick and other species, however, have either made no mention of it or have doubted its existence as a clearly recognizable entity in the chondrocranium.

Another problem of interest is the fate of the preoptic roots of the orbital cartilages in birds. The orbital cartilages in these animals are peculiar in that for a part of embryonic life at least their preoptic roots, the anterior orbital cartilages as they are usually called, are quite separate from their posterior portions which will ultimately ossify to form the latero- or pleurosphenoids. In early stages the anterior orbital cartilages are well developed, resembling the

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condition in many reptiles. They diminish in size rapidly and rather dramatically in the later embryo and, except in so far as they may contribute to the interorbital septum, eventually disappear almost completely. The regression of the orbital cartilages is associated with changes in the relationships of the olfactory nerves which in the adult fowl and many other species pass through the orbits, grooving the sides of the interorbital septum. The nature of these changes is still not entirely clear, especially in the chick, for little work has been done on the chondrocranium of this species for about 50 years.

The present investigation has been undertaken with three main objects:

(1) To describe the development of the interorbital septum in birds with special reference to the distribution and significance of the intertrabecular formation.

(2) To describe and illustrate the fate of the anterior orbital cartilages and the relationships of the olfactory nerves in the chick, and to compare conditions in this species with that in other birds.

(3) To study by experimental methods the possibility that the eyes might exert some mechanical or other influence on the development of the interorbital septum and the structures associated with it.

MATERIAL AND METHODS

The following observations are based on serial sections of thirty normal chick embryos between the 4th and 10th days of incubation, stained either with haematoxylin and eosin or by Masson's trichrome technique. Methylene blue transparencies of six chick embryos ranging between 7 and 10 days were also prepared by the Van Wijhe method. Five sectioned embryos of the sparrow (*Passer domesticus*), ranging between 5 and 11 mm. head-length, have also been available, and for these I am indebted to Mr. M. Abercrombie. Observations on the embryos of other species of South African birds are also discussed. The experimental procedures are described on p. 77.

It should be emphasized that a considerable subjective element enters into the preparation of reconstructions of early stages of the chondrocranium, since large tracts of it are still procartilaginous and their outlines cannot be precisely defined. This is particularly true with bird embryos where so much of the primordial cranium is changing rapidly in shape and disappearing before it ever becomes converted into mature cartilage.

The outlines of the chondrocrania figured are each based on two or more different embryos, displayed by means of graphic reconstructions, transparent paper reconstructions of longitudinal sections (which are useful in studying the almost two-dimensional septal regions of the skull), and in the case of Text-figs. 3 and 5, on methylene blue preparations. Text-figs. 2A and 4 are mainly based on reconstructed models made of waxed blotting-paper.

CONDITIONS IN CHICK EMBRYOS

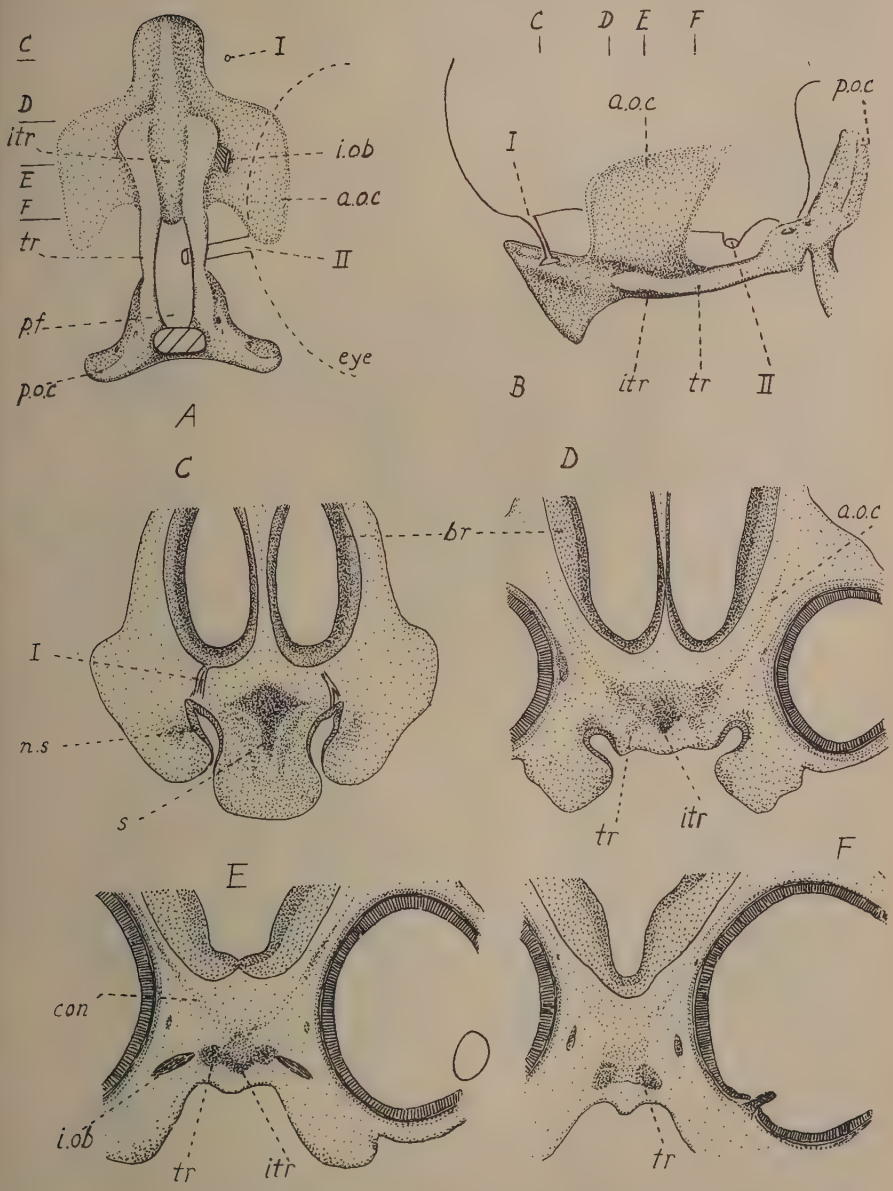
1. *The interorbital septum*

The first appearance of the skeleton in the front of the head can be made out in sections during or rather before the 5th day of incubation. At this time the embryos correspond in appearance with those shown as stage 25 or 26 in the table compiled by Hamburger & Hamilton (which is reproduced in the third edition of *Lillie's Development of the Chick* (1952), edited by H. L. Hamilton. This part of the skull therefore develops slightly later than the acrochordal and parachordal regions.

The nasal and interorbital septum first appears within the tissue between the two nasal sacs as a longitudinal condensation of mesenchyme which becomes more clearly demarcated from its surroundings as one passes backwards towards the eyes. In the posterior nasal region this condensation has a wedge-shaped appearance in section, the tip of the wedge pointing downwards (*s.* in Text-fig. 1C). At a slightly more posterior level (Text-fig. 1D) the mass of condensed mesenchyme broadens out, though it still retains a well-marked median keel. It becomes continuous dorsally on either side with the fainter mesenchymatous condensation lying lateral to the brain from which the anterior orbital cartilage (*a.o.c.*) will later be derived. At this level there appears on either side of the keel a small mass of dark mesenchyme which swells out a little farther back and gives origin to the inferior oblique muscle of the eye (Text-fig. 1E; Plate, fig. C). Here the connexion between the orbital cartilage condensation and the developing septum thins out. Followed farther posteriorly still, the two masses of mesenchyme pass back beneath the optic nerves on either side of the pituitary and join the basal plate (Text-figs. 1 A, B). There can be little doubt that these structures are the trabeculae (*tr*) and polar cartilages which develop in continuity with them.

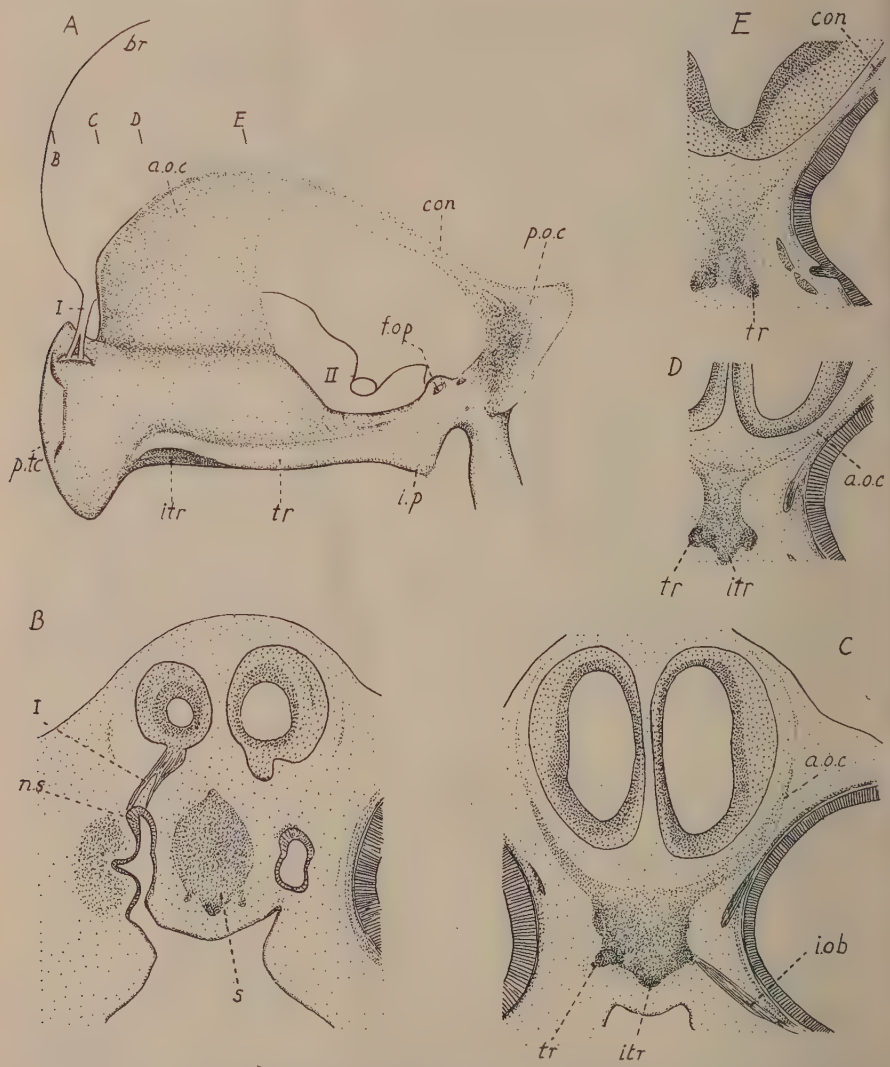
The distinct keel seen in the more anterior sections disappears some distance in front of the optic nerves. It closely resembles the formation described in turtles (Text-fig. 6J) as the intertrabecula, and although it is rather less pronounced in the chick I have labelled it by that name (*itr* in Text-figs. 1 D, E).

In embryos of 6 days (about stages 28 to 29 in the Hamburger–Hamilton table) the appearance of the septum is essentially similar to that in the earlier stage, but it has increased considerably in height (Text-fig. 2A). In the nasal region it seems to consist only of a single median element (Text-fig. 2B); farther back the orbital cartilage condensations extend downwards and inwards to join the trabeculae and the tissue which extends up in the midline between them (Text-figs. 2 C, D; Plate, fig. D). Farther back still (Text-fig. 2E) this tissue seems to make up the greater part of the septum where the anterior orbital cartilages lose contact with its dorsal edge. It is impossible to say whether it has arisen as a condensation *in situ* or has grown upwards and backwards from the keel which is disappearing at this level. It may also have grown up from the trabeculae,



TEXT-FIG. 1. Chick embryo, 5 days. A, ventral and B, left lateral views of chondrocranium with basal plate removed. C–F, transverse sections at levels shown in A and B. (All × 15 approx.) In A a tiny piece of the nasal sac is left attached to the olfactory nerves.

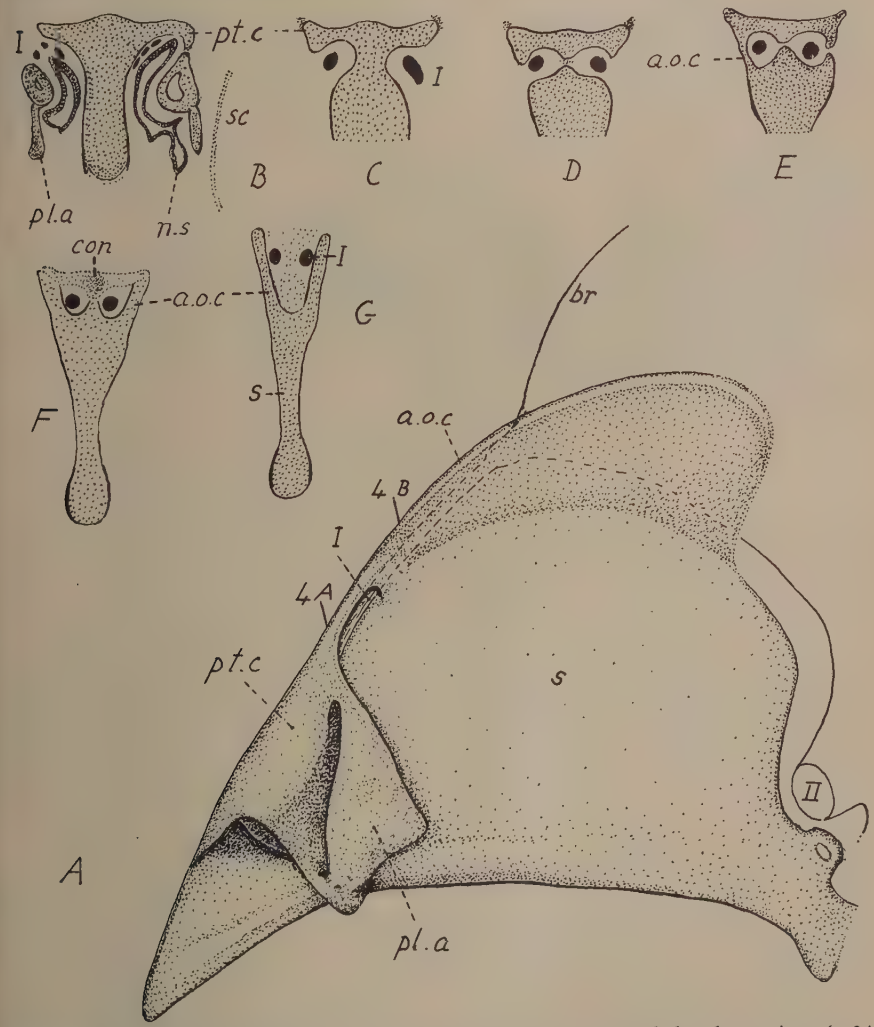
although these still retain a fairly distinct outline and it is possible to pick them out from the mesenchyme of the rest of the septum. A similar appearance is described in the ostrich by Frank (1954).



TEXT-FIG. 2. Chick embryo, 6 days. A, left lateral view of front part of chondrocranium. Lateral part of nasal capsule not shown. A tiny piece of the nasal sac is left attached to the olfactory nerves. B–E, transverse sections at levels shown in A. (All ×18 approx.)

A considerable advance is seen in embryos of 7 days (Text-fig. 3; Hamburger–Hamilton, stages 31–33). The septum is now much higher and relatively thinner,

and the formation of true cartilage, more apparent along the lower edge of the septum than elsewhere, is proceeding. The orbital cartilages have started to chondrify at the sides of the brain.



TEXT-FIG. 3. Chick embryo, 7½ days. A, left lateral view of front part of chondrocranium. (×24 approx.) The lines 4A and 4B correspond with levels of section in Text-fig. 4 A, B. B–G, antero-posterior series of transverse sections through region between lines 4A and 4B, showing olfactory nerve-tunnels. (B–G ×18 approx.)

It is now impossible to distinguish the different components of the septum. The lower edge has lost the three-lobed appearance seen in sections of earlier stages (e.g. Text-fig. 2D) and produced by the median keel with the trabecular

bulges or ridges on either side of it. It is noticeable, however, that the perichondrium over the sides of the swollen lower edge, which are presumably of trabecular origin, stains more deeply than the rest. This appearance is also evident in turtles.

In still later embryos of 8–10 days (Text-fig. 5; Hamburger–Hamilton, stages 34–36) the septum becomes increasingly high and thin and two fenestrae appear in it, one in the posterior nasal region and another, slightly later in the posterior orbital region. These fenestrae seem to arise as in lizards by the absorption of procartilage or young cartilage, and the two layers of the perichondrium fuse to form a sheet of membrane.

By the 10th day much of the septum has become cartilaginous and shows up clearly in methylene blue preparations. Chondrification begins a little earlier in the swollen ventral edge of the septum than it does elsewhere within this structure, but it is impossible to identify discrete centres of chondrification for the parts apparently derived from the various components. The ossification of the septum was not studied.

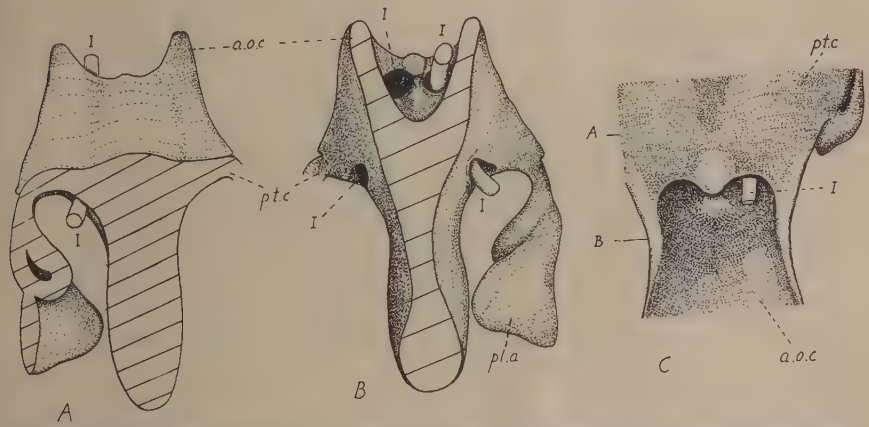
2. *The anterior orbital cartilages and the olfactory nerves*

In embryos of 5 days (Text-fig. 1) the condensations which foreshadow the anterior orbital cartilages (*a.o.c.*) extend outwards from the top of the septum forming a planum supraseptale like that seen in many reptiles. At this stage it is not possible to identify a well-marked connexion between these condensations and those which later form the posterior orbital cartilages. At the 6-day stage (Text-fig. 2A), however, there appears to be a continuous band of condensed tissue connecting the anterior and posterior orbital cartilages (*p.o.c.*), at least in some specimens. This is not evident in later stages and does not develop into a chondrified (though transient) supraorbital cartilage as is the case in certain other birds. Much of the posterior orbital cartilages are still procartilaginous, and as in the earlier stages, it is impossible to reconstruct their outlines accurately.

In the 5- and 6-day stages (Text-figs. 1 and 2) the olfactory nerves (*I*) pass more or less vertically downwards from the brain to the nasal sacs and lie in front of the anterior orbital cartilages and behind the developing roof of the nasal capsule. At or around the beginning of the 7th day, however, this relationship becomes greatly modified and the striking change which takes place seems to occur within a very short time, possibly within 12 hours or less. For this reason the interpretation even of closely staged series is difficult, but the sequence of events, which are not easy to illustrate, seems to be as follows:

The rostral part of the skull lengthens and the beak region approaches the adult outlines, pointing forwards and obliquely downwards (Text-fig. 3A) instead of being recurved as it is in earlier embryos. The parietotectal cartilage (*pt. c.*) grows backwards and its free border on either side appears to fuse with the dorsal and anterior border of the anterior orbital cartilage. At the same time

the septum in the orbitonasal region increases in height and grows up in the midline between the orbital cartilages. Hence the olfactory nerves, which have lengthened very considerably, are each enclosed for a short distance in a tunnel bounded dorsally by the parietotectal cartilage (*pt. c.*), medially by the septum, and laterally by the orbital cartilage (see Text-figs. 3, 4). This tunnel represents the foramen olfactorium evehens.



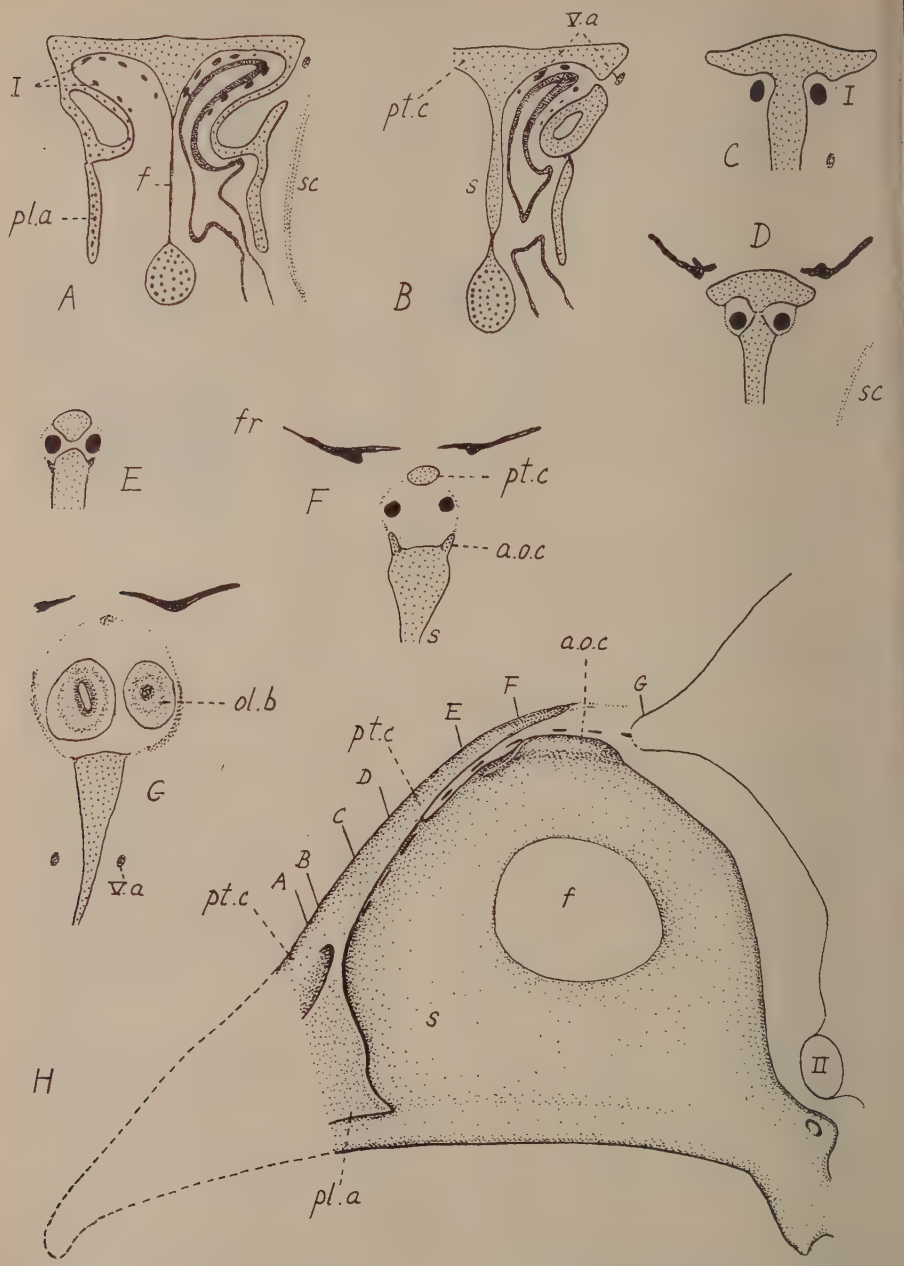
TEXT-FIG. 4. Chick embryo, 7½ days. A, anterior, B, posterior, and C, dorsal views of reconstructed transverse section of region between lines 4A and 4B in Text-fig. 3, showing olfactory nerve-tunnels. Levels of section in 4A and 4B shown in 4C. (×26 approx.)

The changes which occur in later stages are of a more gradual nature. During the 8th day the orbital cartilages diminish in size and in the region where they form the outer walls of the olfactory nerve-tunnels the newly developed cartilage disappears. This regression of cartilage is probably complete by about the 10th day; as it proceeds the intraorbital course of the olfactory nerves lengthens. At the same time the small remaining free portions of the anterior orbital cartilages (*a.o.c.*; Text-figs. 5 F, H) are carried upwards and backwards by the growth of the septum and persist as a small planum supraseptale. This eventually ossifies as the orbitosphenoids.

By about 9 or 10 days the tip of the parietotectal cartilage has grown back above the olfactory nerves and planum supraseptale although the degeneration of the outer walls of the olfactory nerve-tunnels leaves a gap on either side between it and the septum (Text-figs. 5 A, D–G). The conditions shown in a model figured by Tonkoff (1900) of a slightly later stage (6·5 cm. total length) are similar to those described except that the parietotectal process ends some distance in front of the planum supraseptale.

3. *Effects of partial removal of one eye*

Many investigators have suggested that certain features of the bird's chondrocranium, such as the height and thinness of the interorbital septum, the



TEXT-FIG. 5. Chick embryo, 9–10 days. A–G, transverse sections at levels shown in H (×20 approx.). H, left lateral view of front part of chondrocranium after regression of anterior orbital cartilages. (×12 approx.)

regression of the anterior orbital cartilages, and their separation from the posterior orbital cartilages, are in some way related to the great size of the eyes. There is evidence that mechanical factors may play a part in influencing the form of developing tissues, although it is often difficult to demonstrate the existence of such factors in the embryo. It does seem possible that the eyes, anchored as they are by the optic nerves which cross at the chiasma immediately behind the septum, might exert pressure on its formative elements. A relatively faster growth of the eyes as compared with the optic nerves, for example, might conceivably produce some such effect.

Ablation or partial ablation of one eye was therefore carried out either by dissection or with a hot needle through a window made in the egg-shell, subsequently covered with cellophane tape. Most of the operations were performed after 2 or 3 days' incubation. Despite a high operative mortality a number of specimens (10 in all) continued to develop in the incubator and were fixed between the 5th and 14th days of incubation. All attempts to remove both eyes were unsuccessful.

The injured eye regenerated to some extent, usually in a disorderly fashion; it never approached the same size, however, as the normal eye. In two specimens fixed at 7 and 8 days, for example, the regenerated eye rudiment (*e.r.*) had not more than about 5 per cent. of the volume of the normal eye. In such embryos, as other workers have noticed, the beak became crossed, the upper beak deviating towards the side of the injury (Text-fig. 6L).

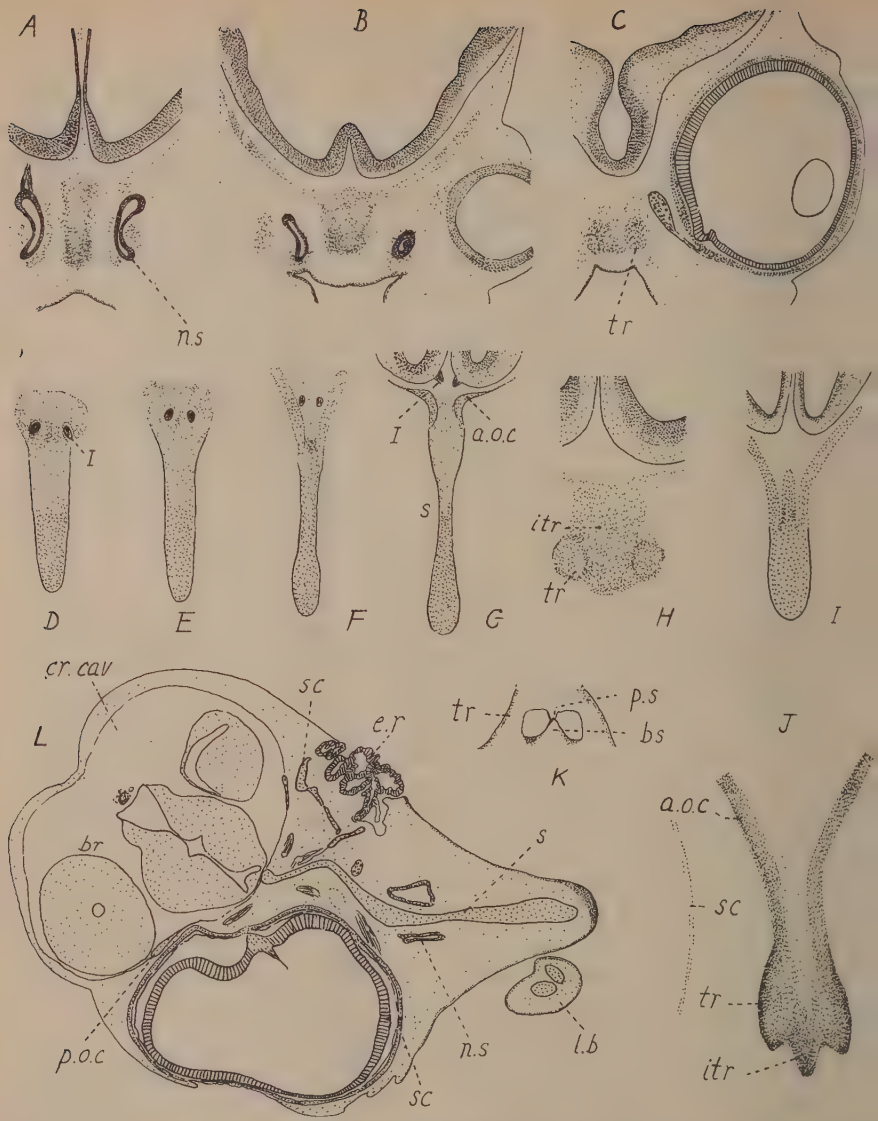
As was reported in a previous note (Bellairs, 1955), the fusion of the various septal components, the regression of the anterior orbital cartilages, and the separation of these from the posterior orbital cartilages were virtually unaffected by the operation. The development and subsequent breakdown of the olfactory nerve-tunnels also took place in the normal way (Plate, figs. B–F). There was a tendency, however, which became more marked in the later stages, for the septum as a whole to become buckled (Text-fig. 6L), as it could hardly fail to do in view of the pronounced asymmetry of the whole head. The anterior orbital cartilage also seemed to be a little smaller on the side of the injury and the upper end of the septum was somewhat asymmetrical (Plate, fig. F).

Conditions in three untreated embryos which were found to show unilateral microphthalmia were essentially similar to those in the operated specimens; such also was the case in a microphthalmic embryo of 7 days obtained by Dr. Ruby Collister as a result of incubating the egg in an oxygen-poor atmosphere (see Collister, 1957).

DISCUSSION

The intertrabecula in Sauropsida

Conditions in the chick may be compared with those in other Sauropsida. In some turtles (Text-fig. 6J) the intertrabecular formation (*itr*) is well developed and forms a wedge between the trabeculae which is more marked than that in



TEXT-FIG. 6. A–C, sparrow embryo, 5-mm. head-length. Antero-posterior series of transverse sections through nasal and interorbital septum (×20 approx.). D–G, sparrow embryo, 9-mm. head-length. Antero-posterior series of transverse sections through interorbital septum showing olfactory nerve-tunnels (×20 approx.). H, *Crocodilus* sp., embryo, transverse section through interorbital septum at front of orbits, early chondrocranium stage. I, ostrich embryo, 15·5-mm. head-length. Transverse section through interorbital septum showing upgrowth between orbital cartilages (after Frank). J, turtle embryo (*Chelydra serpentina*), 5·5-mm. head-length. Transverse section through interorbital septum showing intertrabecula (×30 approx.). K, turtle embryo (*Lepidochelys olivacea*), 26 days. Pituitary fossa region from below showing formation of taenia intertrabecularis from basisphenoid rostrum and process growing back from interorbital septum (after Pehrson). L, chick embryo, 8 days, after previous experimental injury to one eye. Horizontal section through head showing crossing of beak, buckling of septum and regenerated eye rudiment. (×7½ approx.)

the chick. This appearance has been described by Parker (1880) in *Chelone*, by Pehrson (1945) in *Lepidochelys* and *Chrysemys*, and by Bellairs (1949) in *Chelydra*. Parker (1883) described a prominent intertrabecula in Crocodilia and I have recently been able to confirm his findings (Text-fig. 6H). It is probable that the failure of Pehrson (1945) and Bellairs (1949) to identify it in this group was due to lack of the appropriate embryonic stages.

In some turtles the intertrabecula ends in front of the pituitary fenestra, as in the chick. In later embryos of *Chelone* and *Lepidochelys*, however, the fenestra is divided longitudinally by a bar of cartilage which passes beneath the pituitary gland. In Parker's figures of *Chelone* this appears to be continuous with the intertrabecula farther forwards, but Pehrson has shown that in *Lepidochelys* it arises mainly from the forward growth of the basisphenoid rostrum (Text-fig. 6K). This bar across the pituitary fenestra has been called the intertrabecula by some workers; since, however, it may, as in *Lepidochelys*, develop independently from the intertrabecular component of the interorbital septum proper, it seems best to reserve the term taenia intertrabecularis for it (see de Beer, 1937; Pehrson, 1945).

In lizard embryos the intertrabecular formation is not well defined and in my previous study of the interorbital septum (Bellairs, 1949) I did not recognize it as a distinct component of the septum. Re-examination of embryos of the slow-worm (*Anguis fragilis*), however, shows that the trabeculae in the orbital region are joined by a bridge of mesenchyme which in some places has a faintly keel-like shape (*k* in Plate, fig. A).

In snakes, where there is no interorbital septum and the trabeculae remain apart between the eyes, there is little condensation in the intertrabecular mesenchyme. The nodule of cartilage described by Bellairs (1949) in a python embryo is most probably a rudiment of the orbital cartilage system.

The difficulty of interpreting the intertrabecular formation of birds is shown by the rather contradictory accounts which occur. In the chick Parker (1891) briefly states that an intertrabecula is present, but Sonies (1907), whose figures were mainly reproduced from methylene blue preparations, could not recognize it as an independent cartilaginous element. In Lillie's textbook on the development of the chick (1952), however, it is said that 'a high median keel-like plate develops in the interorbital and internasal regions and fuses with the trabeculae, forming the interorbital septum and nasal septum'.

Suschkin (1899) described and figured an intertrabecula in the kestrel (*Tinnunculus*), which seems to resemble that in the chick quite closely. De Beer (1937), however, wrote that 'it is more than doubtful whether this is to be regarded as a distinct element'. Filatoff (1906) also described an intertrabecula in the dove (*Columba*) and figured a schematic diagram in which the trabeculae are shown connected by a bridge of mesenchymatous tissue.

More recent workers on birds have failed to identify an intertrabecular formation. de Beer & Barrington (1934) did not describe it in their careful study of the

chondrocranium of the duck. Crompton (1953) stated that it is absent in the penguin (*Spheniscus demersus*) and from my own study of his material I can confirm that there is no well-defined intertrabecula in this bird. Frank (1954) doubted the existence of an intertrabecula in the ostrich (*Struthio*), although he suggested that a part of the septum which appeared to chondrify independently from the trabeculae and orbital cartilages might correspond with the intertrabecula which Suschkin described in the kestrel. Frank figures, however, a curious upgrowth from the septum between the orbital cartilages (Text-fig. 6I) which I have not observed, at least to the same extent, in other birds.

In my earliest sparrow embryo (5-mm. head-length) the interorbital septum shows in places some indication of paired origin; its ventral part seems to consist of parallel trabecular condensations, close together and partly connected with each other (Text-fig. 6B; Plate, fig. G). There is a suggestion of an intertrabecular keel in the posterior part of the septum (Text-fig. 6C), but elsewhere no distinct keel is present. de Beer (1937) described a taenia intertrabecularis in a sparrow embryo near hatching, but this is not shown in my own younger material.

In the Red Bishop bird, *Pyromelana orix*, conditions are more like those in the chick and an intertrabecular keel is clearly distinguishable though it is less prominent than in the latter species. The chondrocranium of *Pyromelana* is being described by Englebrecht.

It may be concluded that in the early embryonic stages of Sauropsida the trabeculae are separated from each other between the eyes to a greater or lesser extent by a tract of condensed mesenchyme which connects them and stretches above and between them to blend with the orbital cartilage condensations around the brain. In lizards, and birds such as the sparrow and penguin, this mesenchyme is not clearly demarcated from the trabeculae and very soon loses its identity as the trabeculae appear to fuse in the midline and the septum becomes high and thin. In such forms the intertrabecula as a definite structure cannot be said to exist.

In other Sauropsida, however, such as certain turtles and to a lesser extent the chick and the kestrel, the trabeculae seem to lie farther apart at their first appearance and to fuse, not so much with each other as with the bar of mesenchyme between them. In early stages the interorbital septum has the appearance in cross-section of a wedge continuous above with the anterior orbital cartilages and having the trabeculae pressed along each side of its lower edge. Between the trabeculae the mesenchyme extends downwards as a prominent keel so that this part of the septum has a characteristic three-lobed or three-ridged appearance which is still preserved in comparatively late stages (Text-figs. 2 C, D). This wedge of mesenchyme seems to contribute to a large part of the septum and although it is not a separate structure it is a constant and recognizable entity which seems to deserve a special name, the intertrabecula.

Conditions intermediate between those in the two groups described also occur in various species and it is probable that the differences in the appearance of the intertrabecular mesenchyme seen among the Sauropsida are those of degree rather than kind. It is easy to understand how one worker might describe 'an intertrabecula' in a particular species, whereas another, examining a slightly different stage and perhaps using a different staining technique, might state that the structure was absent.

The question remains as to why the intertrabecular formation is so much better developed in some forms than others. It is possible, as Pehrson (1945) suggests, that its prominence may depend on the degree of trabecular separation, and that it is more or less suppressed in forms where these structures develop close together. On the other hand, it is absent in snakes where the unfused trabeculae remain in some species quite wide apart between the eyes. It seems unlikely that the problem as to whether the intertrabecula is a structure in its own right, as it were, or whether its appearance depends on some property of the trabeculae, can be solved by traditional descriptive methods.

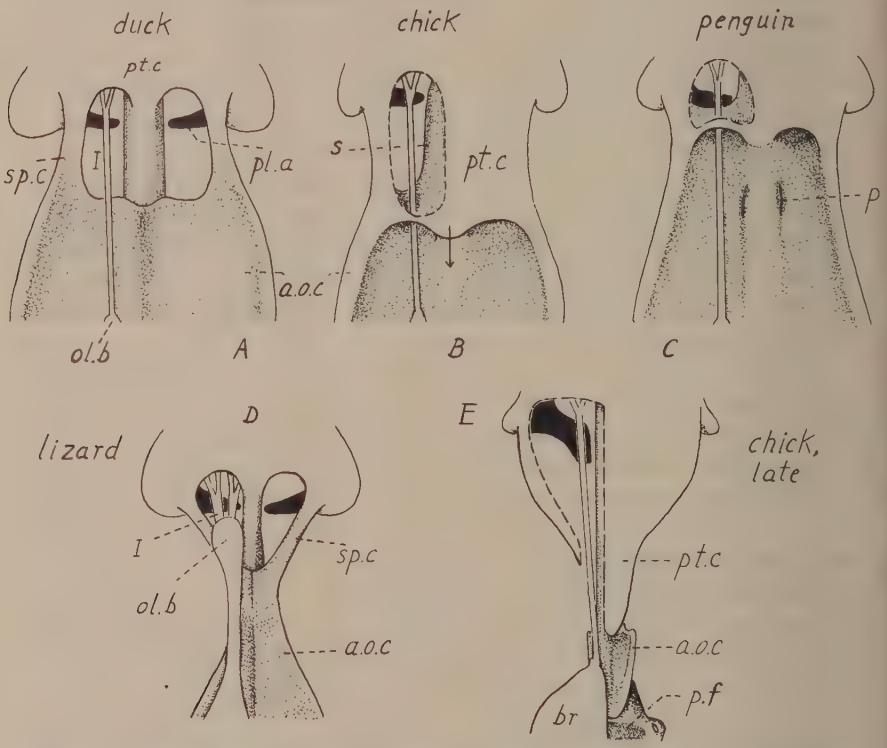
The development of the nasal septum is also of interest. It is generally regarded as a derivative of the fused trabeculae and might therefore be expected to show in early stages some traces of a paired origin. In the chick, however, it appears to arise as a single median structure which is continuous behind with the intertrabecula. Study of the earlier chick embryos suggests that the trabeculae contribute little to the nasal septum for they do not seem to extend much farther forwards than the sites of origin of the inferior oblique muscles at the front of the eyes (Text-figs. 1D, 2C). In the sparrow (Text-figs. 6 A, B), on the other hand, the trabecular condensations seem to pass directly into the ventral part of the nasal septum; since there is hardly any intertrabecular keel they do not show as distinct ridges along the lower edge of the interorbital septum and there is no indication as to where they end in front. No further inferences on the origin of the nasal septum could be drawn from the available material. It is possible that the use of autoradiographic techniques which are said to display procartilaginous mesenchyme at a stage before the onset of histological differentiation (see Amprino, 1955) might throw some light on this problem.

The simple experiments described in this article show that the normal development of both eyes is not necessary for the formation of the interorbital septum and suggest that mechanical pressure of the eyes is not responsible for the coalescence of its various components. This result seems to conform with observations on the development of the skeleton *in vitro* which show that the cartilaginous precursors of bones have a remarkable power of self-differentiation under conditions which are physically abnormal.

The anterior orbital cartilages and olfactory nerves

The formation of olfactory nerve-tunnels is not known in reptile embryos but occurs in the fowl, in the sparrow (Text-figs. 6 D–G), in *Melopsittacus* (Lang,

1955), in the ostrich (Frank, 1954), and probably in many other birds. In the sparrow these tunnels are still present in the full-time embryo (see de Beer, 1937, Pl. 102); it is not unlikely that their outer walls regress after hatching so that finally the olfactory nerves would pass through the orbit in the common avian fashion.



TEXT-FIG. 7. Diagrams showing course of olfactory nerves seen from above in A, duck; B, chick; C, penguin; D, lizard, all at stage of optimum development of chondrocranium, and F, chick at late embryonic stage after backgrowth of parietotectal cartilage and regression of anterior orbital cartilage. Arrow in B shows direction of growth of parietotectal cartilage. In B, C, and E a part of the roof of the nasal capsule, shown by interrupted lines, has been removed. A, based on de Beer & Barrington; C, based on Crompton.

Other variations are seen in the duck and the penguin. In the former the front parts of the anterior orbital cartilages are represented only by narrow bars, the sphenethmoid commissures, much as in lizards (Text-figs. 7 A, D). Furthermore, the roof of the nasal capsule does not grow back above the nerves until a comparatively late stage. The olfactory nerves, therefore, are not enclosed in tunnels and leave the cranial cavity through a large foramen olfactorium evehens on either side of the septum. Later the anterior orbital cartilages regress as in the chick, exposing the nerves still farther in the orbits.

In the penguin short olfactory nerve-tunnels seem to be formed, but they are situated relatively farther forwards than in the chick, so that the olfactory nerves pass directly into the nasal capsule (Text-fig. 7c). The anterior orbital cartilages do not break down, at least not in the stages described by Crompton (1953), so that the nerves do not traverse the orbits. There is, however, little backward growth of the parietotectal cartilage, and the nerves do not become roofed over by its posterior extension, as they are in the later stages of the chick and the duck. The basic similarity between the conditions in the various birds is shown in Text-fig. 7.

The formation of the olfactory nerve-tunnels in birds seems to be due to a temporary excess rather than a deficiency of cartilage in the orbitonasal region, and it seems strange that in these animals so much of the anterior orbital cartilage system should ultimately regress. This regression is, of course, the immediate cause of the exposure of the olfactory nerves, but it may be noted that the reduction of the olfactory fore-brain which has occurred during the evolution of birds is also partly responsible for the condition. In lizards the nerves are relatively short and the olfactory bulbs lie close to the back of the nasal capsule; if the planum supraseptale of the lizard embryo were to disappear it would be the long olfactory stalks and not the nerves which were exposed in the orbits. In birds, however, the olfactory stalks are very short and the bulbs in the adult have retreated far back from the nasal capsule; in order to reach the nose the nerves must be very elongated.

It is reasonable to associate these peculiarities of the avian skull and nervous system with the evolution of the very large eyes which play such an important part in the life of birds. The experimental evidence shows, however, that in ontogeny the normal growth of both eyes is not essential for their development.

SUMMARY

1. The development of the interorbital septum and anterior orbital cartilages in birds has been studied with special reference to the chick embryo.

2. In the chick the trabeculae are at first separated by a bar of intertrabecular mesenchyme which projects downwards between them to form a prominent keel. Later this mesenchyme fuses with the trabeculae and anterior orbital cartilages (preoptic roots) to form the interorbital septum.

3. In some other Sauropsida (i.e. certain turtles and birds) the intertrabecular bar of mesenchyme is also well developed and has a keel. Although this bar is always partly blended with the trabeculae and orbital cartilages, its appearance is sufficiently distinctive to justify the use of the term intertrabecula for it.

4. In other Sauropsida (i.e. lizards, the sparrow) the trabeculae seem to develop closer together and the intertrabecular mesenchyme is less clearly differentiated. The ventral keel is not well developed, and the use of the term intertrabecula is not indicated. The differences between this and the previous group (chick, turtles, &c.) are, however, probably of degree rather than kind.

5. During embryonic life the olfactory nerves in the chick are temporarily enclosed in cartilaginous tunnels formed mainly by the fusion of the anterior orbital cartilages with the roof of the nasal capsule. The subsequent regression of the orbital cartilages is responsible for the exposure of the nerves in the orbits.

6. The different relationships of the olfactory nerves in certain other birds are shown to depend on relatively slight variations of the structure of the orbitonasal region of the chondrocranium.

7. The formation of the interorbital septum, the growth and regression of the anterior orbital cartilages and the disposition of the olfactory nerves are virtually unaffected by the experimental ablation or partial ablation of one eye at a stage before the onset of skull development. Conditions similar to those in the operated specimens were found in three chick embryos which showed naturally occurring, unilateral microphthalmia.

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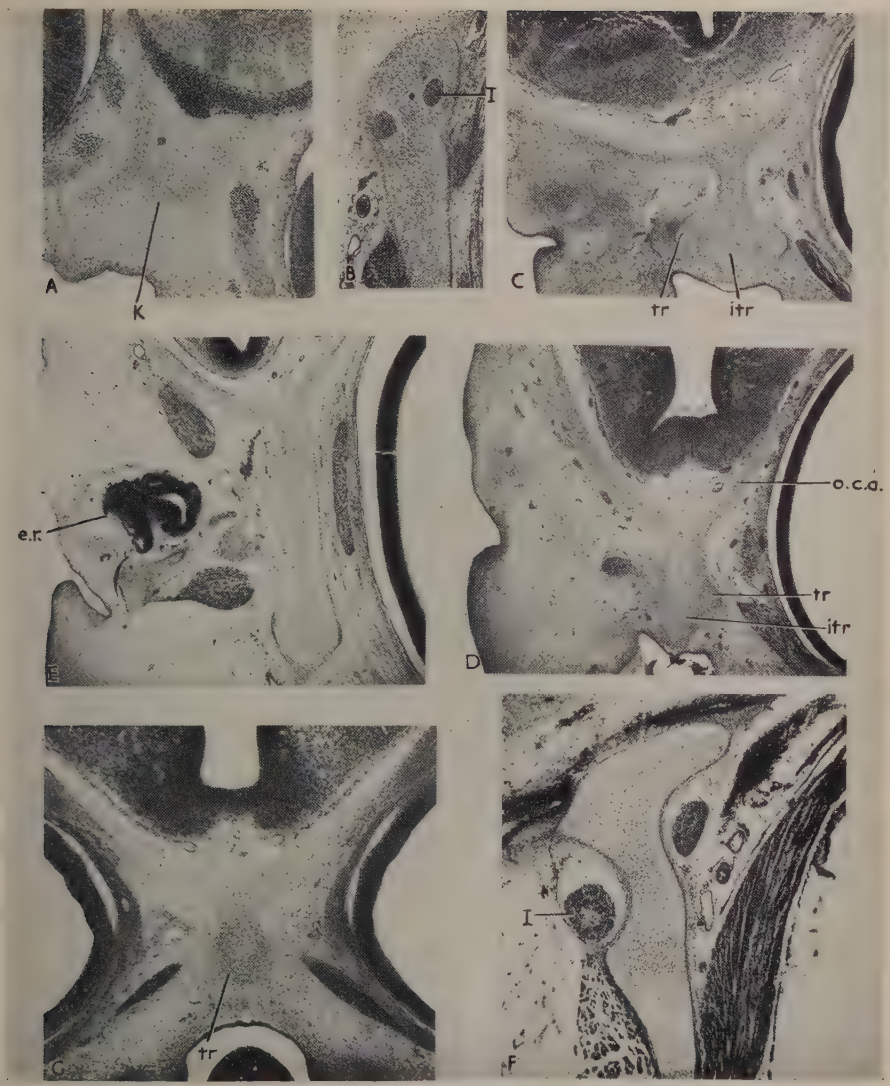

Transverse sections through interorbital septum

A. Lizard (*Anguis fragilis*, 3·6 mm. head-length embryo). Early stage showing slight intertrabecular keel (*k*) (×70 approx.).

B. Chick, 8½ days showing formation of olfactory nerve-tunnels after ablation of eye on left side (of photo) (×26 approx.).

C, D, E. Chick, 5½, 6, and 7 days respectively showing formation of septum after ablation of eye on left. E shows regenerated eye rudiment; C and D are in front of the level of the rudiment (×28 approx.).

F. Chick, 14 days after ablation of eye on left. The upper edge of the septum is asymmetrical. The outer walls of the olfactory nerve-tunnels have regressed (×38 approx.).

G. Sparrow (*Passer domesticus*, 5 mm. head-length embryo). Level near front of eyes showing poorly differentiated intertrabecular mesenchyme. The orbital cartilage condensations do not reach the septum at this level (×34 approx.).

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ABBREVIATIONS IN TEXT-FIGURES AND PLATE

a.o.c., anterior part of orbital cartilage.
br., brain.
bs., taenia intertrabecularis of basisphenoid.
con., orbital cartilage condensation.
cr. cav., cranial cavity.
e.r., eye rudiment.
f., fenestra in septum.
f. op., foramen for ophthalmic artery.
fr., frontal.
i. ob., inferior oblique muscle.
i.p., infrapolar process.
itr., intertrabecula.
k., intertrabecular keel.
l.b., lower beak.
n.s., nasal sac.
ol. b., olfactory bulb.
p., perforation in orbital cartilage.
p.f., pituitary fossa.
pl. a., planum antorbitale.
p.o.c., posterior part of orbital cartilage.
p.s., process from interorbital septum.
pt. c., parietotectal cartilage.
s., nasal and interorbital septum.
sc., sclera.
sp. c., sphenethmoid commissure.
tr., trabecula.

I, olfactory nerve and tunnel.
II, optic nerve.
Va, ethmoidal and ophthalmic nerves.

(Manuscript received 17 : v : 57)

Comparative Effects of Moni-, Di-, and Triphosphorylated Nucleosides on Amphibian Morphogenesis

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INTRODUCTION

EXPERIMENTS previously reported (Ambellan, 1955) established the fact that short-term treatment of *Rana pipiens* blastulae with solutions of mononucleotides under specific conditions resulted in accelerated formation of neural tubes: at 18° C. they were about 4 hours ahead of controls. As briefly mentioned in the previous paper, slight overdoses or excessive periods of treatment resulted in visible side-reactions affecting other aspects of development in conjunction with the precocious neural tube formation. A comparison of the different effects that result from overdoses of adenosinemonophosphate (AMP-3), adenosinediphosphate (ADP), adenosinetriphosphate (ATP), and other compounds that differ from each other only in the number of phosphate bonds may throw some light on the biochemical nature of the morphogenetic processes differentially affected by these substances and is the subject of this paper.

MATERIALS AND METHODS

A homogeneous population of dejellied embryos was selected and groups raised each in about 15 ml. of solution in covered Stender dishes in a constant temperature water-bath at about 18° C. Compounds under investigation were added to 10 per cent. Ringer's solution, then brought to pH 5·6–5·8 with Ringer's hydroxides (a combination of K, Na, and Ca hydroxides with the same cation concentrations as in Ringer's solution). Controls were at the same pH.

'Optimum conditions' were arbitrarily defined as the concentration of substance and duration of treatment at which there were no apparent effects on the embryo other than on neurulation; the embryos developed normally to hatching. These generally were: between 12–24 hours of treatment with ATP 1 mM. or with AMP-3 1 mM., and up to 8 mM. of ADP or IDP (inosinediphosphate), although there was some clutch-to-clutch variability in concentrations tolerated.

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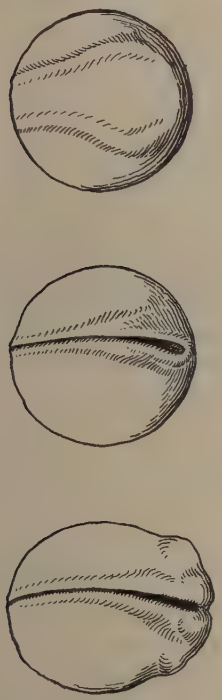
RESULTS

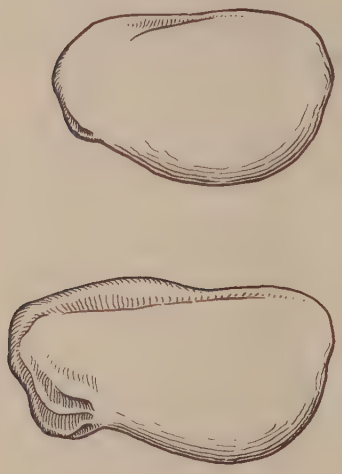
Treatment of *R. pipiens* embryos with solutions of ATP, AMP-3, ADP, IDP, or mixtures of AMP-3 + ATP and mixtures of other diphosphorylated nucleosides accelerated neural-tube formation under 'optimum conditions'. The morphogenetic acceleration was of the order of 6 hours at 16° C., 4 hours at 18° C., or 3 hours at 23° C. With excessive but sub-lethal concentrations of materials or



TEXT-FIG. 1. Effect of mononucleotides on neural-tube closure. All animals from same clutch raised under constant temperature conditions. *Top*: control, open neural plate (stage 14). *Middle*: ATP-treated embryo, closed neural tube (stage 15), defective sense plates. *Bottom*: ADP-treated embryo, closed neural tube, sense plates normal.

duration of treatment specific morphogenetic effects of the several nucleotides were observed in addition to early neural-tube closure (see Text-fig. 1). The development of head and gill structures (Shumway stages 16 and 17) immediately following neurulation in normal morphogenesis was blocked with overdoses of ATP, but not with overdoses of any other substance used (see Text-fig. 2). With overdoses of AMP-3 the first visible defect in morphogenesis was a block in the growth of the tail-bud (Shumway stage 18). At similar concentrations ADP or IDP had no adverse effects, and development following precocious neurulation was apparently further stimulated, although the advance over controls was difficult to measure objectively. A typical experiment in which treatment was continuous, beginning during the blastula stage, is reported in Table 1.

The effects of the mononucleotides were morphologically specific for the process of neural-tube closure in that regardless of the time of treatment there were no visible differences between control and experimental animals during blastula,



TEXT-FIG 2. Effect of mononucleotides on head-structure development. Both animals are the same chronological age. *Top*: ATP-treated embryo showing microcephaly. *Bottom*: control embryo at stage 16. ADP- and AMP-3 treated embryos resemble controls.

TABLE 1

Comparative effects of AMP-3, ADP, and ATP on frog morphogenesis

Substance in solution (mM.)	*Observation times after fertilization at 18° C*		
	60 hours	*72 hours*	*96 hours*
10% Ringer (controls) . . .	Open neural plate*	Head structures develop†	Tail-bud growth‡
ADP 4·0	Closed neural tube§	+	+
AMP-3 4·0	Closed neural tube	+	Stunted
ATP 4·0	Closed neural tube	Microcephalic	Stunted

+ *Apparent* advance over controls; head and gill structures often enlarged at 72 hours, tail-buds often longer and general improved viability compared to controls at 90 hours.

* Shumway stage 14.

† Shumway stage 16.

‡ Shumway stage 19.

§ Morphological resemblance to Shumway stage 15.

gastrula, or early neural-plate stages. However, while controls then required about 8 hours (18° C.) to completely close the neural tubes, experimental animals formed completed neural tubes in 4 hours. Eggs treated at the 2-cell stage with ATP (as suggested by Dr. L. G. Barth) or with ADP, left overnight at 8° C. and washed thoroughly before being returned to Ringer's solution, showed no differences from controls during blastula or gastrula stages, but exhibited precocious neurulation, which occurred 2–3 days after removal of the eggs from the

treatment solutions. In most of the experiments treatment was started during blastula stages, but it was also effective when applied as late as stage 14 when embryos have flat, open neural plates.

Some differences were noted in the rate of neural-tube closure between the effective substances. When stage-14 embryos were treated at 23° C., the neural tubes closed in about 45 minutes with ATP solutions, in about 1 hour with ADP, and in about $1\frac{1}{2}$ hours with AMP-3. Controls took about 3 hours to completely close the neural tubes at this temperature. In experiments in which substances had been applied 2 days prior to neurulation and in which the precise onset of tube closure happened to be observed, the process started first in the ATP group, about 20 minutes later in the ADP group, and about 30 minutes after that in the AMP-3-treated embryos.

Specific effects of ATP treatment

Side effects observed with overdoses of ATP, occurring with an intensity proportional to concentration, were: delayed closing of the blastopore, microcephaly following precocious neurulation, and, finally, lack of development of neural ridges, gill plates, and sense plates at the time of tube closure (Text-figs. 1 and 2). Some or all of these abnormalities were noted in over forty experiments using ATP solutions and were never observed with overdoses of any other materials used. These specific results of ATP treatment did not generally appear when equimolar AMP-3 was added to the ATP solutions. The ATP experimental animals were bloated and stunted by stage 18, but these later defects were also observed with AMP-3 and ADP treatment.

Specific effects of AMP-3 treatment

With precocious neurulation resulting from AMP-3 the development of neural ridges, sense, and gill plates was apparently normal. Following neurulation embryos often showed grossly exaggerated head and gill structures, but never microcephaly. The first block in development with overdose treatment did not appear until the tail-bud stage when embryos were stunted as with ATP treatment; but they were less severely so, and recovery upon return to Ringer's solution was more frequent. These after-effects from AMP-3 treatment were more variable than with other substances used and occasionally did not appear at all. It had previously been found that AMP-5 did not consistently advance neurulation under these same conditions and it was not used in these investigations.

Effects of ADP, IDP, and other diphosphorylated nucleosides

There were no visible side effects or after effects from ADP or IDP when used at the same concentrations that had proved toxic with ATP or AMP-3. The advanced developmental rate observed during neurulation apparently continued through the following stages when head structures and tail-buds develop, and embryos often showed improved viability compared to controls. An ADP concentration about four times that of ATP or AMP-3 was required to produce any

abnormal effects. These abnormalities then resembled the AMP effects described above. Head structures were normal or exaggerated, and stunted tail-buds were the first defects to appear.

Embryos treated with equimolar solutions of AMP-3 + ATP generally resembled those treated with ADP alone, although there was some individual

TABLE 2

Comparative and concentration effects of di- and triphosphorylated nucleosides on frog morphogenesis

Substance in solution (mM.)	*Observation times after fertilization at 20° C*		
	48 hours	*60 hours*	*72 hours*
10% Ringer (controls) . . .	Open neural plate	Head structures develop	Tail-bud growth
ADP: 0·25, 0·5, 1·0, 4·0, and 8·0 (5 separate dishes)	Closed neural tubes	+	+
ADP 0·75 + IDP 0·25 . . .	„ „	+	+
ADP, CDP, GDP, UDP: 0·25 each	„ „	+	+
ATP 4·0	„ „	Microcephalic	Stunted
„ 1·0	„ „	Like controls*	Stunted
„ 0·5	„ „	Like controls	Stunted
ATP 1·0 + AMP-3 1·0 . . .	„ „	+	Like controls
„ 0·5 + „ 0·5 . . .	„ „	+	Like controls**

\+ Apparent advance over controls, head and gills often larger.

* Two embryos in this group were microcephalic.

** One-half of the ten embryos in this group were microcephalic.

No apparent variability occurred between ten embryos in each of the other experimental groups or four different groups of controls.

variability within experimental groups not found when ADP alone was used. Although treatment with 4 mM. ATP resulted in typical microcephaly, treatment with a mixture of ATP + AMP-3, 4·0 mM. each, resulted in normal head development with no apparent abnormalities until tail-bud stage. Embryos treated with this mixture sometimes cytolysed at about stage 19, which did not happen when ADP alone was used.

IDP can effectively replace ADP in this system. In an experiment using this material (kindly supplied by Dr. S. Ochoa) at 8, 4, and 1 mM., both continuously and for 12-hour treatments started at blastula stages all embryos in all the experimental groups showed $4\frac{1}{2}$ hours advance in neural-tube closure. By the tail-bud stages embryos treated at the highest concentration (8 mM. continuously) had stunted bodies, and those treated at the lowest concentration (1 mM. for 12 hours) resembled controls. Embryos in the other four experimental groups (1 and 4 mM. continuous, and 4 and 8 mM. 12-hour treatments) lived for 8 days while three different groups of controls died in 6 days under the slightly overcrowded conditions of this experiment. Embryos from this same clutch of eggs treated continuously with ATP at 1 and 4 mM. cytolysed immediately after the precocious neurulation.

Combinations of ADP with uridinediphosphate (UDP), guanosinediphosphate (GDP), and cytosinediphosphate (CDP) were effective in advancing neurulation, but no more effective—at the lower ranges of concentrations used—than was ADP alone. Results of experiments carried out with Dr. Herman Kalckar at the National Institutes of Health (Bethesda, Md.) are reported in Table 2. Treatments were continuous starting at blastula stages. Solutions were at pH 5·6–5·8.

DISCUSSION

The original hypothesis that ATP in solutions might serve as an energy source in morphogenesis had to be modified when it was found that formation of the neural tube—the first structure formed in morphological differentiation in amphibia as in all vertebrates—was accelerated by all of the mononucleotides, including AMP-3 which does not have the labile, 'high energy' phosphate bond. This part of the results generally supports Brachet's hypothesis (1947) on the role of mononucleotides in morphogenesis, although in these experiments the *visible* effects were on neural-tube and not on neural-plate formation. While precocious neurulation was produced by any of the mononucleotides, the rate of this acceleration was directly proportional to the number of phosphate groups on the nucleotides used: fastest with ATP, next with ADP, and slowest with AMP-3.

The formation of head and gill structures follows neural-tube formation in normal development. Hörstadius (1950) has shown that neural-crest cells that appear at the time of neurulation are concerned in the formation of these structures. This developmental system is apparently affected by treatment with solutions of ATP. Even mild overdoses result in microcephaly by stage 16, and with larger overdoses this microcephaly is foreshadowed by the lack of visible neural ridges, sense, and gill plates during the time of rapid neural-tube formation. Treatment with AMP-3, ADP, IDP, or other diphosphorylated nucleosides, never blocked neural-crest or head-structure development at any concentrations used. Embryos, in fact, often showed exaggerated formation of these structures, and it may be that this next morphogenetic process, as well as neurulation, can be enhanced with overdoses of these substances.

The diphosphorylated nucleosides were the most innocuous materials used to advance neurulation inasmuch as other developmental processes were least disturbed. This suggests that the mechanism by which these materials are utilized to advance neurulation may more closely resemble normal synthetic mechanisms of the embryo than utilization of ATP or AMP-3. The details of the dose effectiveness reported in Table 2 also suggest that utilization of the mono- and triphosphorylated nucleosides may be by way of a kinase catalysing the reaction

$$2\,\mathrm{ADP} \rightleftharpoons \mathrm{ATP} + \mathrm{AMP},$$

since the abnormal effects of ATP were largely reversible by the addition of equimolar AMP-3 to solutions.

While there is no direct evidence in these experiments for penetration of the effective materials, three aspects of the results make this a likely assumption: (*a*) the pH dependence of the results, (*b*) the reversibility of the specific morphogenetic effects of ATP treatment by addition of AMP-3 to treatment solutions, and (*c*) the clear separation in time between application and effect of treatment, especially in experiments in which the visible effects appeared 2–3 days after removal of embryos from treatment solutions and in which ultra-violet spectrophotometric readings confirmed that all free, unbound nucleotides had been removed.

The possibility that the mononucleotides may be utilized metabolically, either directly or indirectly, in the synthesis of key materials is now under active investigation.

SUMMARY

1. Treatment of *Rana pipiens* embryos with adenosinetriphosphate (ATP), adenosinemonophosphate (AMP-3), adenosinediphosphate (ADP), and other diphosphorylated nucleosides, either under 'optimum conditions' or with an overdose, resulted in formation of neural tubes about 4 hours (at 18° C.) ahead of untreated controls. With overdoses specific side-reactions appeared that differed with the substance applied.

2. The rate of neurulation was directly related to the number of phosphate groups of the nucleotides used; it was fastest with ATP, intermediate with ADP, and slowest with AMP-3. The other side-reactions varied specifically and consistently with the number of phosphate groups, but the intensity of these reactions was not directly proportional to the number of phosphate groups present.

3. Overdose treatment with ATP blocked development of head and gill structures immediately following precocious neurulation. This effect was largely reversible by addition of equimolar AMP-3 to solutions.

4. Overdose treatment with AMP-3 apparently enhanced formation of head and gill structures. Development was first blocked at the tail-bud stage.

5. ADP, IDP, and other diphosphorylated nucleosides at similar concentrations had no adverse effects following precocious neurulation and apparently stimulated growth of the entire embryo.

6. All of the mononucleotides specifically accelerated neural-tube formation whether applied at the 2-cell stage, during the blastula stage, or at the stage of the open neural plate.

ACKNOWLEDGEMENTS

In addition to the assistance acknowledged in the text, I would like to express thanks to Drs. Z. Dische and Alfred Gellhorn for laboratory hospitality during

these past two years; to Dr. France Baker for the attached drawings; to Drs. Eric Hirschberg, Alan Wachtel, and S. Ochoa for manuscript suggestions; and to Dr. Herman Kalckar whose active interest in this work was indispensable.

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The Effects of Homologous Testicular and Brain and Heterologous Testicular Homogenates combined with Adjuvant upon the Testes of Guinea-Pigs

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WITH ONE PLATE

NUMEROUS efforts have demonstrated antibody production after injection of spermatozoa into animals of the same species. For example, Metalnikoff (1900) and Kennedy (1924) reported anti-guinea-pig sperm 'toxins' in guinea-pigs; McCartney (1923) noted anti-rat sperm 'toxins' in rats; Pfeiffer (1905), Dittler (1920), and Pommerenke (1928) demonstrated anti-rabbit 'spermatotoxins' in rabbits. Antibody production against heterologous sperm has also been disclosed: Mudd & Mudd (1929) injected human, guinea-pig, bull, and ram sperm into rabbits and reported that the resultant antibodies were species specific. The absoluteness of specificity, both organ and species, however, has been qualified by the study of Lewis (1934), who found that brain and testicles possess common antigens, and Henle (1938) has extended Mudd & Mudd's (1929) observations on cross-reaction between sperm of closely related species. In the above-mentioned studies the methods for determining antisperm activity of antisera included complement-fixation, sperm-immobilization, agglutination, and precipitin tests.

Recently, Freund, Lipton, & Thompson (1953) employed adjuvants combined with homologous and autologous testicular homogenates to induce aspermatogenesis in guinea-pigs. The report of Freund and co-workers is intriguing, since the ability to induce a loss of tissue either by destruction of existing tissue or by prevention of proliferation is tantamount to control of such fundamental processes as growth and differentiation. In the case of induced aspermatogenesis it appears that only the spermatogenic elements are affected. The opportunity to study such a unique response seemed an exciting one, and was accentuated by reports that active immunization with brain (Freund *et al.*, 1947) and with thyroid (Rose & Witebsky, 1956) can cause damage in the respective homologous organs. These conditions (aspermatogenesis, encephalomyelitis, and thyro-

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pathy) are presumed to be effected by an auto-immune response. If this type of reaction should be found to apply to other organ systems, it might reveal an underlying concept of broad and fundamental significance; namely, that each tissue or organ might contain antigens which, under proper conditions, could lead to the morphological and/or functional alteration, and perhaps even to the destruction, of the organs from which the antigens are derived. This current study initiated efforts directed at investigating this concept.

After confirming the results of Freund *et al.* that homologous testicular preparations in adjuvant do induce aspermatogenesis in guinea-pigs, a broad programme was undertaken to gain information on the following points: (1) Is the antispermatogenic factor restricted to the seminiferous epithelium or does the nonspermatogenic portion of the testis also contain the agent? (2) Can heterologous testicular preparations or homologous brain homogenates be employed in similar fashion to affect spermatogenesis? (3) Is the reaction by which the testes are damaged an immunological process?

This report indicates that an antispermatogenic factor resides in the spermatogenic tissue alone; that heterologous testicular preparations and homologous brain tissue do induce lesions in the testes; and that the weight of evidence is in favour of an immune basis as the mechanism whereby the testis is damaged.

MATERIALS AND METHODS

Forty-nine adult male guinea-pigs were treated as noted in Table 1. They were fed a complete diet including greens, and fresh water was available at all times. All of these animals gained weight and were apparently free of infection. Eight other guinea-pigs lost weight or developed infection during the experiments, and these animals were, therefore, excluded from this report.

A weighed amount of the material to be injected was homogenized in a Ten Broeck (1931) grinder with an equal volume of a 0·9 per cent. solution of NaCl. Aliquots of the homogenates were emulsified in equal volumes of the Freund complete adjuvant (paraffin oil, Bayol F, and *Mycobacterium butyricum*; Freund, Lipton, & Thompson, 1953) with the aid of a syringe and a blunt-tipped 15-gauge needle. Between 0·5 and 1·0 ml. of the emulsion was injected intracutaneously in several (5–15) sites close to the nuchal region and along the mid-dorsal line. When several courses of injection were made, approximately 2 weeks intervened between successive administrations. The animals were examined frequently, at which times the testes were palpated; severe testicular damage could be noted by reduction in size of the organ. Body-weights were recorded at each injection and at sacrifice. At the time of sacrifice, blood was withdrawn by cardiac puncture, and the sera were stored in a deep-freezer for later use; the testes were weighed, and fragments for biopsy, together with pieces of seminal vesicle and prostatic tissue, were placed in Bouin's picro-formol or 10 per cent. neutral formol solution. In order to check the presence and motility of sperm,

the epididymides were flushed of sperm by forcing 0·9 per cent. saline through the vas deferens using a 22-gauge needle and a 5-ml. syringe and slitting the

TABLE 1

The effects of injections of homologous and heterologous testicular homogenates and other materials on the testes of guinea-pigs

Guinea-pig number	*Injected material* (mg. per injection)*	*Number of injections*	*Number of days after first injection when sacrificed*	*Weight of both testes (g.)*	*Testis damage rating†*
		Guinea-pig testis			
58	Guinea-pig testis (50)	2	37	3·0	2
94–97	Guinea-pig testis (50)	2	41–47	2·3, 2·0, 0·76, 1·54	3, 3, 4, 3
10–11	Guinea-pig testis (75)	3	64	0·87, 1·3	4, 4
30–33	Guinea-pig testis (100–150)	5	91	2·9, 1·0, 1·8, 2·2	3, 4, 4, 4
82–85	Guinea-pig testis (100–250)	5	70, 70, 203, 169	1·7, 2·0, 3·4, 1·5	4, 4, 3, 4
134–5	Guinea-pig testis (250)	1	114	1·2, 1·0	4, 4
		Damaged guinea-pig testis			
60–65	Damaged guinea-pig testis (100–200)	6	80–85	6, 5·5, 4·6, 4, 4, 4	0, 0, 0, 1, 0, 1
		Guinea-pig brain			
20–22	Guinea-pig brain (100–200)	4	94	1·6, 2·8, 4	3, 2, 1
		Heterologous testis			
66–67	Rooster testis (100–225)	5	75, 125	3, 3·2	2, 1
69	Rooster testis (100) 3 Human ejaculate (0·25 ml.) 2 Bull testis (250) 1	6	75	2·6	2
71–72	Human ejaculate (0·25 ml.) Human ejaculate (0·25 ml.) 2	5	75, 124	2·5, 3·2	2, 1
73	Rooster testis (100) 2 Bull testis (250) 1	5	75	1·5	4, 3
81	Rat testis (300)	4	62	3·5	0
952, 955, 973, 978	Rooster testis (100) 2 Human ejaculate (0·25 ml.) 2 Bull testis (250) 1	5	97–89	2, 3·4, 4, 3·5	4, 1, 1, 1
122, 124	Monkey testis (60–250)	2, 3	61, 97	2·4, 3·5	3, 1
		Control injections			
13, 15	Freund adjuvant alone (1 ml.)	4	67	3·6, 3·8	0, 0
78–79	Histidine (10)	5	70	3·3, 3·6	0, 0
86–87	Hyaluronic acid (15)	4	107	3·7, 3·5	0, 0
98–99	Galacturonic acid (20)	4	71	4·0, 4·0	0, 0
		Guinea-pig testis in saline			
56–57	Guinea-pig testis in saline I.V., I.P., S.C.‡ (125–250)	9	50	3·6, 3·7	1, 1

* All materials were incorporated with Freund complete adjuvant unless otherwise stated.

† Ascending numerals indicate increased magnitude of damage as described in Materials and Methods.

‡ I.V., I.P., S.C. refer to intravenous, intraperitoneal, and subcutaneous injections respectively.

epididymides when pressure had bulged the tubules. The sperm so obtained were examined microscopically for motility and density and were used immediately for sperm-immobilization and agglutination studies, or were stored in a deep-freezer for subsequent complement-fixation tests.

Histological examinations were made on sections of the organs, cut at 7 μ and stained with hematoxylin-eosin or Heidenhain's azan.

Histological interpretation of the testicular biopsies was based upon a rating scale in which ascending numerals indicate increasing order of damage in a manner similar to that utilized by Freund *et al.* (1953). Testicular injury was rated as follows: 0 represents the normal or control condition; 1 indicates a depletion of mature sperm and the presence of sloughed cells in the lumina of the seminiferous tubules; 2 refers to an increase in cellular material and debris in the lumina and a vacuolation of the cytoplasm in the primary spermatocytes; 3 refers to a general lack of secondary spermatocytes and the exfoliation of primary spermatocytes; 4 is the extreme condition in which there may be a complete or an almost complete absence of spermatogenic elements, including spermatogonia, which imparts to the tubules the appearance of vacuoles lined by Sertoli cells with or without their processes.

For complement-fixation tests the sera were inactivated at 60° C. for 3 minutes and absorbed with sheep cells. A block titration against sperm was carried out using fresh or frozen-thawed guinea-pig sperm according to the method of Osler, Strauss, & Mayer (1952). For sperm-immobilization tests, 0·1 ml. each of diluted serum, guinea-pig complement, and fresh sperm were combined in glass depression slides and observed during a one-hour period.

OBSERVATIONS

Histologic results

Injections of guinea-pig testis plus adjuvant. Thirty-seven days after the injection of guinea-pig testis (animal No. 58), testicular injury was found corresponding to the type-2 condition (Plate, fig. A). In four guinea-pigs (94–97) permitted to survive 41–47 days after the first of two injections, a variable but increased damage was observed. The extent of damage ranged from impaired testes with masses of exfoliated and necrotic cells in the lumina to a condition with sparse spermatogenic tissue and the absence of material in the lumina. In tubules which still retained some intact cell constituents, sperm-tails without heads were seen in the tubules. The cytoplasm of the primary spermatocytes was highly vacuolated and agranular, and the nuclei were condensed and distorted. In tubules containing few cells, vacuolization of the cytoplasm was so pronounced that cell borders and pycnotic nuclei only were observed. In the most advanced stage of degeneration observed at this time, only Sertoli cells and their processes, with but few isolated spermatogonia and primary spermatocytes, were present. With longer times, 64–169 days, and with a greater number of injections (3–5) the extent of injury was consistently severe (guinea-pigs Nos. 10, 11, 31, 32, 33, 82, 83, and 85). These tubules included a few primary spermatocytes and spermatogonia and a fair population of Sertoli cells whose wavy processes appeared prominently in the lumina. However, even the spermatogonia

and Sertoli cells were reduced in number, so that the lumina appeared as vacuoles (Plate, fig. B). In these extreme cases interstitial tissue appeared more prominent than in the normal testis.

Individual variation was encountered in the appearance of tubules within a testis and in testes of animals which received the same treatment. The testes of guinea-pig No. 30, for example, were not so severely damaged as were those of Nos. 31–33, all of which were injected 5 times and carried for 91 days. Moreover, in some instances a more extensive injection schedule was followed by a less severe testicular response. Thus the testes of guinea-pig No. 30 (injected 5 times during 91 days) were not so severely injured as were those of guinea-pig No. 10, which received only three injections during 64 days. Also it was not infrequent that one testicle was more gravely injured than was the other. These differences may reflect a difference in individual sensitivity on the part of the animal as a whole and of the testis. Nevertheless, as appears from the data in Table 1, the total time between injection and sacrifice is important, for guinea-pigs Nos. 134 and 135 received only one injection each during 114 days, and their testes were maximally affected.

A time factor may also be important in an entirely different respect, namely, in the reversibility of the lesions with subsequent recovery of spermatogenesis. Four guinea-pigs (Nos. 82–85) were injected 5 times with guinea-pig testis during 63 days. On the 70th day animals No. 82 and No. 83 were found to have suffered severe testicular damage. Guinea-pigs No. 85 and No. 84 were permitted to survive for an additional 99 and 133 days respectively. The testes of guinea-pig No. 85 were found to be as profoundly damaged as were those of guinea-pigs Nos. 82 and 83. On the other hand, the degree of damage in guinea-pig No. 84 was considerably less than that in any of the others in this group. Many tubules were sterile, yet several had all stages including secondary spermatocytes, and a few tubules even possessed mature sperm. Whereas recovery may thus be possible after induced aspermatogenesis, the series is too small to justify a conclusion in this regard.

Damaged guinea-pig testis plus adjuvant. Histological examination of the testes of guinea-pigs Nos. 30–33 (Table 1) revealed that the treatment had induced severe depletion of the spermatogenic elements. These damaged testes were then homogenized and emulsified with complete adjuvant in the same manner as the unaffected testes had been prepared in the previous series. Six animals (Nos. 60–65) were then injected on six different occasions with this material over a span of 80–85 days (Table 1). The following observations were made. At sacrifice the testicular weights of all six animals were in the normal or control range, 4 to 6 g. (Table 1), and epididymal sperm were as numerous and as motile as those found in control animals. Extensive histological examination of approximately 200 sections of the testes of all six guinea-pigs disclosed that in no instance had damage beyond the type-1 stage occurred, and this slight impairment was observed in only two animals (Nos. 63 and 65). This effect

referred to the occurrence of cells and debris in the lumina of some few tubules, but even in these tubules spermatogenesis had not been arrested. All of these findings (testicular weight, sperm motility and density, and histological data) indicate that the spermatogenic capacity of these animals injected with damaged testis was essentially unaffected by this treatment (Plate, fig. C).

Injections of guinea-pig brain plus adjuvant. Four guinea-pigs (Nos. 20–23) were injected with homologous brain emulsified in complete adjuvant. Four series of inoculations were given during 94 days (Table 1). One of the animals (No. 23) lost weight, and for that reason its data are not included. The testicle weights of the other three guinea-pigs at sacrifice were as follows: 1·6, 2·8, and 4·0 g. for Nos. 20–22 respectively. Histological examination disclosed that the epididymides of guinea-pig No. 20 contained much debris, which included fragmented sperm and spermatogenic tissue. The degree of injury in the testes of this animal was variable but generally was severe; damage ranged from that in tubules, in which only spermatogonia and Sertoli cells could be seen, to a condition in a few tubules where secondary spermatocytes were encountered. The abundance of tubules which contained no stages beyond the primary spermatocyte, and the necrobiotic appearance of these cells, indicated a type-3 reaction (Plate, fig. D). A variable but generally less severe response than that of guinea-pig No. 20 was found in the testes of animals No. 21 and No. 22. The injury observed in the former case was predominantly of type-2 wherein few or no mature sperm were in the tubules, spermatids were lacking, and the kind as well as the amount of debris in the lumina indicated sloughing of the primary spermatocytes. In the testes of guinea-pig No. 22 little evidence of mature sperm or spermatids could be found, but the depletion of spermatocytes was less severe than that observed in the other animals in this series.

Injections of heterologous testis plus adjuvant. Two guinea-pigs, Nos. 66 and 67, were injected 5 times with rooster testis during 75 and 125 days respectively. The lesions in the spermatogenic tissue were moderate, namely, types-2 and 1 respectively. Again, the injury was not uniform throughout the testes but varied from tubule to tubule. A similar destructive effect was observed in two animals which received five injections of human ejaculate during 75 and 124 days respectively. The injection of rat testis 4 times during 62 days (guinea-pig No. 81), however, produced no damage. Two or three injections of rooster testis, one injection of bull testis, and two injections of human ejaculate into six guinea-pigs (Nos. 69, 73, 952, 955, 973, and 978) led to varying degrees of damage which ranged from a mild effect to severe destruction. In animal No. 73 one testicle was affected maximally and the other had a type-3 response (Plate, figs. E, F).

Two guinea-pigs responded to injections of monkey testis. Animal No. 122 received two injections and after 61 days had a type-3 injury; No. 124, after 97 days, showed a type-1 condition.

Control injections. In Table 1 it can be seen that guinea-pigs which received four or five injections of complete adjuvant alone or in combination with

histidine, hyaluronic acid, or galacturonic acid over a period of 67 to 107 days had testes that were indistinguishable from those of untreated animals selected at random from the colony. Freund *et al.* (1953) have previously shown that certain other organ preparations (liver and kidney) plus adjuvant had no effect on the testes.

Saline homogenates of guinea-pig testis. Two guinea-pigs (Nos. 56 and 57) were injected with saline preparations of guinea-pig testis not combined with adjuvant. The material was given intravenously, intraperitoneally, and subcutaneously. The testes of these animals were found to have suffered a slight injury (type-1); cellular debris and exfoliated cells were present in some of the lumina, but the majority of tubules were unaffected.

Accessory organs of reproduction. The seminal vesicles and prostatic glands of the treated animals were not different from those of the controls. At autopsy these glands were observed to be normal in size and to contain abundant secretion. Histological examination confirmed the fact that these organs of the treated guinea-pigs were indistinguishable from those of untreated animals.

Serological observations

Complement-fixing antibody titers were found when the sera of guinea-pigs injected with homologous testis were incubated with guinea-pig sperm. No complement-fixing antibodies, on the other hand, were detected in the sera from guinea-pigs which received adjuvant alone. It was later found in the studies with rabbits that high titers of antisperm complement-fixing antibodies were evoked by injections of homologous sperm, even though no lesions were observed in the testes of these animals. Consequently the relationship between complement-fixing antibodies and testicular damage must be viewed with caution.

The sera of guinea-pigs sensitized with homologous testis plus adjuvant were found to immobilize 50 per cent. of fresh sperm within one-half hour. This inhibition of motility was frequently accompanied by head-to-head, tail-to-tail, and indiscriminate agglutination. The results obtained with precipitin, agglutination, and ring tests were inconsistent, and therefore these methods had to be abandoned.

DISCUSSION

One of the goals of the current investigation was to determine whether the antispermatogenic factor is a property of the spermatogenic tissue or is found also in the nongerminal portions of the testis as well. The answer is provided by the results obtained on the animals that were injected with testis in which only remnants of germinal tissue remained as a result of previous immunization with homologous testicular extracts. The lack of damage, despite six separate injections during 80 to 85 days, points clearly to the fact that the antispermatogenic factor resides in the gametogenic tissue. Another important fact is demonstrated

by these experiments in which damaged testis was injected, namely, that androgenic substance present in the testes cannot be responsible for the lesions. The androgenic activity of the damaged testis was not different from that of undamaged testis as judged by the size and histological condition of the seminal vesicles and prostatic glands. The injection of damaged testis had no significant effect on the testes of the recipient animals.

Another aim of the present work was to determine whether heterologous testicular homogenates and homologous brain tissue could induce injury in the spermatogenic tissue. The results obtained with rooster or monkey testis, human ejaculate, or homologous brain extract indicate that these materials do induce injury. The fact that the lesions so obtained are not so severe or as uniform as are those evoked by homologous testicular tissue may be a reflection of the fact that interspecific cross-reactions are rarely so strong immunologically as are intraspecific ones (Mudd & Mudd, 1929; Henle, 1938). Brain–testicle interactions are also weaker than are those between the same organs. These points have been made clear by Lewis (1934) and Henle (1938), who referred to such interactions as species and organ 'selectivity' rather than the more rigid species and organ 'specificity'. Freund *et al.* (1953) failed to induce aspermatogenesis in the guinea-pig after injecting bull sperm or testis from the rabbit, hamster, or sheep. They concluded that the reaction was species specific. It may be that the heterologous testicular materials employed by these investigators were less closely related serologically to the guinea-pig than are those tissues obtained from the monkey and man. The same workers also recorded only mild testicular lesions following injections of homologous brain preparation, and they concluded that the mechanism of damage may be unrelated to that induced by testis, since loss of body-weight was observed in these animals. In the current work, however, testicular lesions induced by brain homogenate were observed in healthy, growing animals. Thus, the probability of brain–testicle interaction is strongly indicated.

With regard to the third objective of these studies, the available evidence supports the view that the testicular damage results from an immune response. Lewis (1934) demonstrated that antisera against the testis of a given species reacted most strongly with the testes of individuals of the same species; cross-reactions were weaker between such antisera and the testes of animals from closely related species; and no serological reactions occurred with the testes of distantly related animals. These principles should be applicable to aspermatogenesis if the process by which it is produced is an immune one. The evidence indicates that this is so. In the current work guinea-pig testis was more effective in causing damage in guinea-pig testicles than was monkey and human testicular material, which, however, did give positive reactions. In the work of Freund *et al.* (1953), aspermatogenesis was unobtainable by testicular material from bull, rabbit, hamster, or sheep. The results with brain injections also support the view that aspermatogenesis is induced by an immune reaction. Henle (1938) provided evidence that brain and testicle have a common antigen. In the present

studies brain injections did cause testicular damage. The most likely interpretation is that the injury was due to an immune response. The work of Lewis (1941) also supports this interpretation, for he revealed that brain and testicle reacted with each other but did not react with liver, kidney, heart, lung, or spleen. Consequently if aspermatogenesis is induced by an immune mechanism, only testis or brain material should cause this effect. This appears to be true, for Freund *et al.* (1953) have shown that liver and kidney were unable to damage guinea-pig testes.

Further and compelling evidence for an immune reaction is presented in the current work. If testicular material damages selectively only the germinal elements of the testes and not the nongerminal portions, then the antigenic material should be found only in the spermatogenic tissue. This report points clearly to this selectivity, since guinea-pigs' testes damaged by treatment with homologous testis and then injected into other recipients induced no significant injury.

The mild damage induced in the testes of guinea-pigs No. 66 and No. 67 by rooster testis is a curious result, since cross-reactions between two such distantly related forms is unusual. This finding implies some common antigenicity shared by rooster and guinea-pig testes. The problem is being investigated further.

The principal objection against an immune mechanism as the causative factor in aspermatogenesis was raised by Freund *et al.* (1953). These workers injected a 'mitochondrial' fraction obtained from guinea-pig testes and found that, while severe damage was present in the testes of the treated guinea-pigs, there were no significant complement-fixing antibody titers in the sera of these animals. It is entirely likely, however, that complement-fixing antibody titers do not reveal the true nature of the immune response. It is to be recalled that injections of homologous testes into rabbits caused a significant elevation in complement-fixing antibody titers, but no damage was seen in the testes of these animals. Other methods of measuring antibody levels in guinea-pigs injected with testis were no more revealing than was the complement-fixation method. The clues to the demonstration of the immune mechanism responsible for destruction of the spermatogenic tissue may be that the response is an allergic one. In such cases, there is an apparent lack of correlation between circulating antibody titer and hypersensitivity (Ehrenkranz & Waksman, 1956). However, induction of aspermatogenesis by means of transfer of cells must be explored as a possibility before a strong position can be taken with regard to the mechanism involved.

SUMMARY

1. In confirmation of the work of Freund *et al.* (1953), it was found that intracutaneous injection of an emulsion of guinea-pig testis and complete adjuvant resulted in damage to the spermatogenic tissue of recipient guinea-pigs. The degree of damage varied from mild to severe. In the moderately injured testicles

sperm were absent, spermatogenesis was disrupted, and exfoliated germinal cells were found in the lumina of the tubules; in severe injury most germinal elements, including spermatocytes and spermatogonia, were severely depleted, and frequently only Sertoli cells and few spermatogonia remained. The degree of damage is a function of time as well as of sensitivity of the individual animal. The suggestion is made, further, that partial recovery of spermatogenesis may occur if the damage is not too severe and sufficient time is allowed to lapse after injection.

2. The antispermatogenic factor resides in the spermatogenic tissue and not in the nongametogenic portions of the testes.

3. Androgen present in the testicular homogenates is not responsible for the lesions in the guinea-pig's testes.

4. The seminal vesicles and prostatic glands of all recipient guinea-pigs remain normal, which clearly demonstrates that the treatment has no effect on androgenic activity.

5. Testicular tissue from roosters and monkeys, human semen, and guinea-pig brain, each incorporated into adjuvant, also induced spermatogenic lesions. These materials were, however, less efficacious in evoking as uniform and severe a lesion as consistently resulted from injections of guinea-pig testis. These findings are explainable on an immune basis, since reports in the literature concerning interspecific cross-reactivity indicate the weaker nature of such interactions when compared with intraspecific reactivity. Reports also indicate that brain and testicle share a common antigen. The precise relation of the immune response to the induction of aspermatogenesis remains to be revealed.

ACKNOWLEDGEMENTS

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EXPLANATION OF PLATE

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FIG. A. Animal No. 58. Mild damage in seminiferous tubules 37 days after first of two injections of homologous testis. Note luminal debris and vacuolated spermatocytes.

FIG. B. Animal No. 33. Severe damage 91 days after first of five injections of homologous testis. Only spermatogonia and Sertoli cells remain.

FIG. C. Animal No. 60. No damage in testis 80 days after first of six injections of damaged homologous testicles. This gonad is indistinguishable from normal, control testes.

FIG. D. Animal No. 20. Injury induced by homologous brain. Seminiferous tubules lack stages beyond primary spermatocyte. Animal sacrificed 94 days after first of four injections.

FIGS. E, F. Animal No. 73. Seminiferous tubules 75 days after first of five injections of heterologous testicular material (human ejaculate, rooster, and bull testes). Damage in left gonad is maximal and in right testis is less severe.

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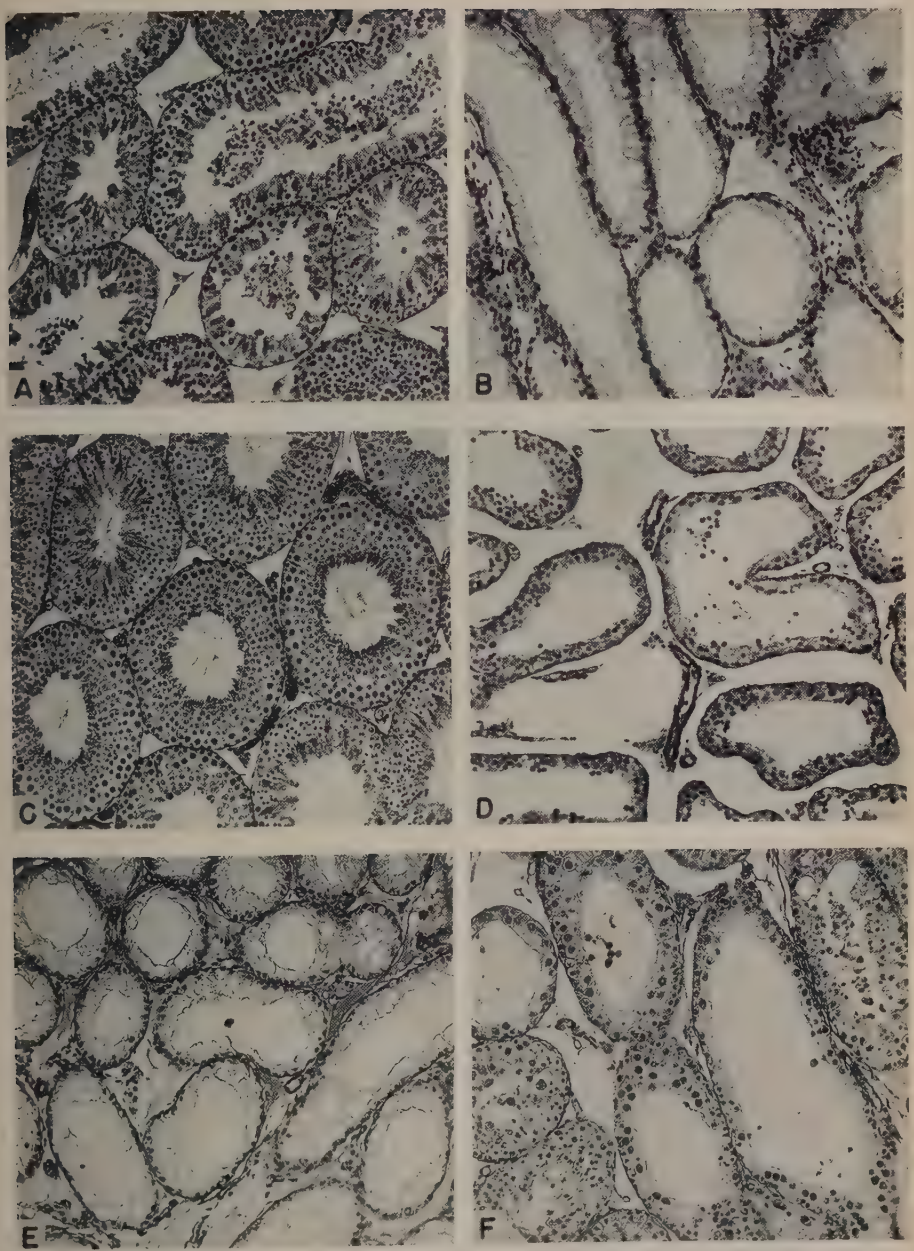
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Twirler: A Mutant Affecting the Inner Ear of the House Mouse

by MARY F. LYON[1]

From the M.R.C. Radiobiological Research Unit, A.E.R.E., Harwell

WITH ONE PLATE

THE mutant *twirler*, symbol *Tw*, of the house mouse, *Mus musculus*, was first recognized by the 'waltzing' behaviour which the heterozygotes show. It was later found to affect the morphology of the inner ear and, when homozygous, to cause death of new-born animals through harelip and cleft palate.

This paper describes the behaviour and ear defects of heterozygotes and the appearance of homozygotes. It also describes the genetic tests carried out to determine the mode of inheritance.

DESCRIPTION

Heterozygotes

Twirler heterozygotes show head-shaking in a horizontal plane, combined often with circling, and less often with vertical head-shaking. Postural reflexes are abnormal. For example, normal mice of about a week old, if held up by the tail, respond to this change of position by extending the back and stretching the forelimbs forward. Twirler mice, on the other hand, flex the back, tuck the head under, and draw the limbs back. The 'landing reaction', in which the forelimbs are extended powerfully forwards in response to a downward falling movement, is also absent. In non-twirlers it develops during the third week of life.

Most twirlers react normally to sound. In animals where no reaction to sound can be observed it is difficult to tell whether the animals are truly deaf or are merely failing to stop circling long enough for a reaction to be seen.

The growth of young animals is usually normal although a few twirlers lag behind their normal sibs in growth. Adults, on the other hand, tend to become obese. This obesity may become obvious at any age from 3 months onwards, but some twirlers live out a normal breeding life and remain slim. The fat is found in the usual sites for deposition of fat in the mouse. Subcutaneous fat is particularly obvious in the inguinal and neck regions and between the shoulder blades. The 'hibernating gland' between the shoulder blades seems normal in

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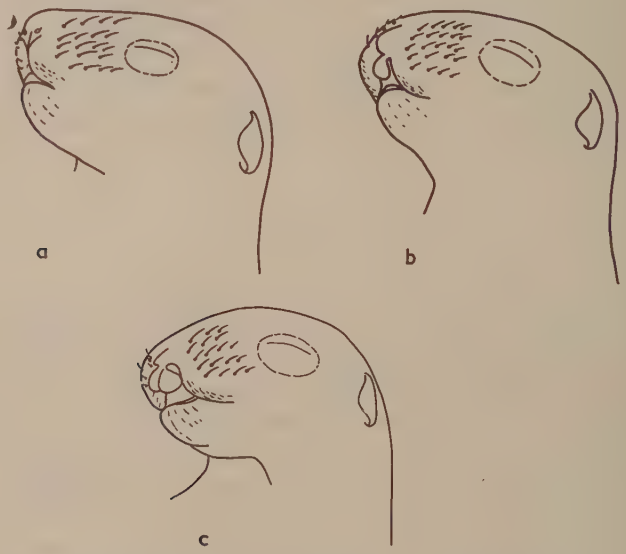
size. In the abdomen fat surrounds the kidneys, ovaries, and testes, and fills the mesentery.

Both sexes are fertile but become sterile if obesity sets in. Some twirlers make good parents but some animals of both sexes are liable to attack and kill the young at any time during the first week of life, more especially after being disturbed in some way, e.g. by being given a clean cage. Male twirlers, if allowed to grow up together, will often begin to fight when about 2 months old to such an extent that they must be either separated or destroyed.

Homozygotes

Animals considered to be homozygous have shown either cleft lip and palate or cleft palate only. Of 30 such animals observed at birth, 13 showed cleft lip and palate and 17 had normal lips with cleft palate. Among the animals with cleft lips the defect was bilateral in 7 cases, on the left only in 2 cases, and on the



TEXT-FIG. 1. Heads of new-born mice. *a*, normal; *b* and *c*, twirler homozygotes. *b* shows a left-sided cleft lip and *c* a bilateral cleft lip. In *b* the left and in *c* both maxillary processes have failed to grow to the midline, leaving the medial nasal processes exposed.

right in 2 cases (Text-fig. 1). In the remaining 2 cases the side affected was not noted. Cleft palate was usually median or bilateral, these two categories not being separable, but in 2 cases it occurred on the left only and in 2 on the right only. One of the left-sided clefts accompanied a left-sided cleft lip and in the other unilateral cases the lip was not cleft.

All homozygotes died within 24 hours of birth. The more highly affected ones died of respiratory difficulty. When removed from the mother by Caesarean section animals with cleft palate and harelip could be stimulated to breathe regularly by the same massage and pinching which would stimulate normal mice. When the stimulation was stopped, however, and the animals were left to breathe spontaneously they gradually became cyanosed. If again stimulated, so that they squeaked and gasped, they would become pink once more, but when the stimulation was stopped they again became cyanosed and died within a few hours. Animals without harelip and with a relatively narrow cleft in the palate were able to breathe normally, but if placed with a foster mother they died within 24 hours, presumably from starvation. Unaffected sibs born by Caesarean section and placed with foster mothers fed and grew normally. Some animals with harelip and cleft palate swallowed large amounts of air into the stomach and intestines. It is not clear whether this air was swallowed in attempts to suck or to breathe.

The inner ears of homozygotes are abnormal but have not yet been fully investigated.

EAR DEFECTS OF HETEROZYGOTES

Methods

The ears of twirler heterozygotes were studied by means of whole mounts of the bony labyrinth and of serial sections of the complete inner ear.

To prepare whole mounts of the bony labyrinth, part of the skull including the labyrinth was fixed in 70 per cent. alcohol for some days, macerated in 1 per cent. potassium hydroxide until the adherent soft tissue became transparent, dehydrated in alcohol, and cleared in benzyl alcohol. No stain was used. The bony labyrinth was then dissected away from the surrounding bone.

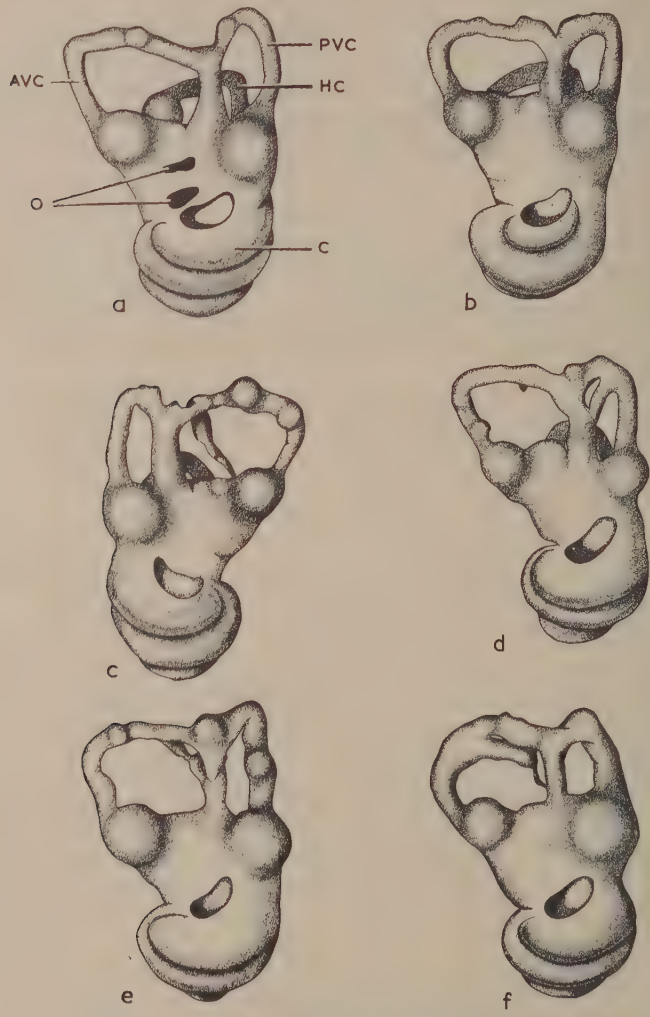
For sections of the inner ear 3- to 4-week-old mice were fixed by the injection of Bouin's fluid into the aorta. The inner ear was removed and further fixed in Bouin's fluid, decalcified with 2 per cent. nitric acid in 70 per cent. alcohol, and embedded in 3 per cent. celloidin and paraffin by the methyl benzoate-celloidin technique. Transverse sections were cut at 7–10 μ, and stained with haematoxylin and eosin.

Observations

(*a*) *Whole mounts.* Text-fig. 2 shows some examples of abnormalities found in the bony labyrinth. The typical defect consisted of reduction or absence of the horizontal canal, accompanied by absence of otoliths, and uneven outlines of the vertical canals. The cochlea appeared normal.

The degree of defect varied considerably from one animal to another. At the least the horizontal canal was present and only slightly reduced in length, as in Text-fig. 2*a*, and the otoliths were present, although sometimes consisting of fewer crystals than normal. Some ears had one otolith present and one absent.

Ranging from this slight defect there were progressive stages of reduction of the horizontal canal, accompanied by complete loss of otoliths, and by increasing



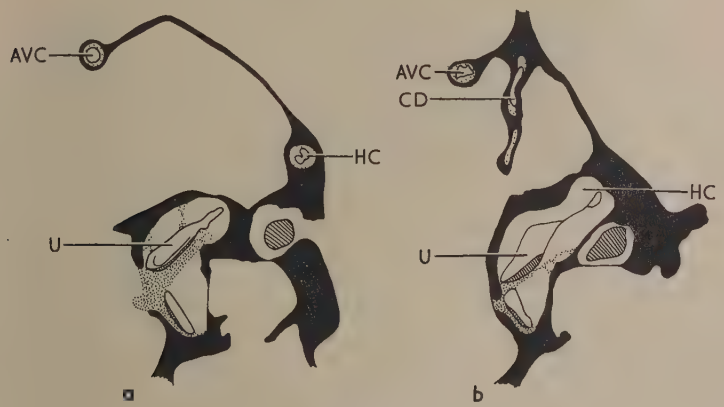
TEXT-FIG. 2. Camera lucida drawings of the bony labyrinths of twirler heterozygotes, showing increasing grade of defect. *a* differs from normal only in the uneven outline of the vertical canals, while *f* lacks otoliths, has no visible horizontal canal, and shortened and branched vertical canals. AVC, anterior vertical canal; C, cochlea; HC, horizontal canal; O, otolith; PVC, posterior vertical canal. × 10.

abnormality of the vertical canals. The minimal defect of the vertical canals was unevenness of outline. The unevenness then increased to form projections from

the walls of the canals. In the most extreme cases the projections formed complete branches or duplications which ran from the canal to the utriculus or the crus commune (Text-fig. 2 *c*, *f*). The arcuate fossa, in which the floccular lobe of the cerebellum normally lies, might be more or less obliterated by branches from the anterior vertical canal. Or the fossa might be smaller than normal as a result of reduction in length of the vertical canals.

(*b*) *Sections.* Serial sections were used to study the histology of the membranous labyrinth. Eighteen ears of 9 twirlers were examined and compared with 7 ears of normal sibs.

In the utriculus and sacculus the otoliths were usually lacking (Plate), but in the position in which they would normally be found there was some material which showed the same staining properties as, and was considered to represent, the organic matrix of the otolith. The neuro-epithelium of the maculae over which the matrix lay showed no abnormality and the remainder of the utriculus



TEXT-FIG. 3. Camera lucida drawings of transverse sections of the ears of a normal mouse (*a*) and a twirler heterozygote (*b*). In *b* the horizontal canal is shortened so that it has no separate bony capsule, and there is a canal diverticulum which, in other sections, could be seen to run from the anterior vertical canal to the utriculus. AVC, anterior vertical canal; CD, canal diverticulum; HC, horizontal canal; U, utriculus. ×20.

and sacculus appeared normal. When otoliths were present they were in some cases thinner than usual. Of the 18 ears examined, 9 lacked both otoliths, 7 had a normal saccular otolith, in 3 cases accompanied by a thin utricular otolith, and the remaining 2 ears had a thin saccular otolith only.

In the horizontal canals both the crista and the morphology of the canals were abnormal. The canal itself was always shortened and sometimes its bony capsule was not separated from that of the utriculus, so that in preparations of the bony labyrinth it would have appeared that the horizontal canal was lacking

(Text-fig. 3). In fact, none of the 18 ears examined entirely lacked a horizontal canal or ampulla. The ampulla, probably in association with the shortening of the canal, was somewhat most posterior in position than in normal mice, and the crista lay lateral to rather than anterior to the utricle macula. It was always abnormal in shape. Normally the horizontal crista forms an upfolded ridge of epithelial cells extending across the ventroposterior wall of the ampulla. In twirlers the epithelium forming the crista was folded inwards rather than up, forming a pit instead of a ridge, in the ventromedial part of the ampulla (Plate. fig. A). The histological differentiation of the neuro-epithelial cells seemed normal, and covering material which probably represented the cupula was present.

The ampullae and cristae of the vertical canals were normal, but the lumen of the canals themselves varied in diameter, as was to be expected from the unevenness of outline seen in whole mounts of the bony labyrinth. In some cases the lumen extended into the diverticula of the canals (Text-fig. 3), but in other cases where the diverticula were narrower they contained only a solid core of connective tissue cells, with no canal lumen.

In all except two animals the cochlear duct was completely normal. In one of these two animals the left cochlear duct was normal but the right was highly hydropic, the organ of Corti was disorganized, and the stria vascularis apparently absent. The sacculus in this ear contained some form of pink-staining precipitate. In the other animal, a litter-mate of the first, both cochlear ducts were affected. The basal region appeared normal but the apical region was hydropic, with the organ of Corti abnormal. In the right ear the stria vascularis seemed absent from this region; in the left ear it was normal.

In some twirler ears the lumen of the endolymphatic duct appeared wider than in the normal sibs, suggesting some excess of endolymphatic fluid. It is not clear whether this was of much significance, however, as the lumen may vary in normal animals and the distension in twirlers was slight.

GENETICAL STUDIES

The mutation occurred spontaneously in a crossbred multiple recessive stock homozygous for the genes *a*, *b*, c^{ch}, *d*, *s*, *se*, the first twirlers found being two females which occurred in two successive litters of 8 born to a certain pair. Later studies showed that these females must have been heterozygous for a dominant gene, and hence it may be presumed that the mutation that gave rise to them occurred in the germ-line of one or other parent.

Of the two original twirlers one failed to breed and the other produced 11 offspring by her brother, 4 twirler and 7 normal. Four normal sisters of the twirlers were mated to the sire or a brother and produced a large number of young, all normal. This preliminary evidence suggested that the new character was due to a dominant gene, a hypothesis which was confirmed by further work.

When twirlers were mated together the ratio of twirler to normal offspring was closer to a 2 : 1 than a 3 : 1 ratio, suggesting death of homozygotes before classification.

Single-factor ratios

The numbers of twirler and normal offspring obtained in various types of cross are shown in Table 1. Twirler heterozygotes were outcrossed to linkage-testing stocks and to various inbred strains and the *Tw*+ × ++ class in the table includes these outcrosses and also the subsequent backcrosses of *Tw*+ offspring to the linkage stocks. The animals mated in the *Tw*+ × *Tw*+ class were the progeny of outcrosses.

TABLE 1

Single-factor ratios of twirler

Type of cross	Progeny		Expected	χ^2
	Tw	+		
Outcross . . . *Tw*+ × ++	466	558	1:1	8·3
Intercross . . . *Tw*+ × *Tw*+	84	58	2:1	3·6
Tests . . . *Tw*+?× ++	155	143	1:1	..

After *Tw*+ × ++ matings the proportion of twirler offspring is significantly less than the expected half ($P < 0{\cdot}01$); and with *Tw*+ × *Tw*+ matings the ratio of twirler to normal offspring is less than the expected 2 : 1 but not significantly so ($0{\cdot}05 < P < 0{\cdot}1$). Such a deficiency could be caused by incomplete penetrance of the twirler gene in the heterozygous state or by death of twirler offspring before classification. From observation of the animals either explanation seems possible. The grade of behaviour defect in twirler heterozygotes is variable and it is possible that some low-grade animals have passed undetected. On the other hand, highly affected twirlers are sometimes small and thin at $2\frac{1}{2}$ weeks, when classification was usually made, and it is possible that some highly affected animals died before this age. In either case the deficiency is small; if incomplete penetrance is the cause then the penetrance may be estimated from the outcrosses as 2 × 466/1024 or 91·0 per cent., and if low viability is responsible for the deficiency, then the relative viability of twirlers may be estimated as 466/558 or 83·5 per cent. The degree of deficiency in the intercrosses is similar to that in *Tw*+ × ++ matings and the penetrance may be estimated as (84 × 3)/(2 × 142) or 88·7 per cent. or the viability as 84/2 × 58 or 72·4 per cent.

Homozygotes

In order to test the hypothesis that no *TwTw* young live, twirler young of intercross matings were tested for the possibility of their being *TwTw* by mating to unrelated normal animals. Of twenty-four such animals tested all produced at least one normal offspring and were taken to be *Tw*+. The numbers of twirler and normal offspring resulting from these tests are shown in Table 1. The

proportion of twirlers is somewhat higher than in the outcrosses, which suggests that some of the tested animals may in fact have been homozygous *TwTw* but, owing to incomplete penetrance of twirler, did not produce all twirler offspring. On the basis of the outcrosses and intercrosses such animals would be expected to throw about 90 per cent. of twirler offspring. In fact none threw ratios of twirler to normal at all unexpected for the 1 : 1 of heterozygotes, so that there is no positive evidence that *TwTw* animals ever live and breed.

TABLE 2

Frequency of cleft lip and cleft palate among the offspring of twirlers

Mating		No. of litters	Offspring				
Series	Type		Normal	Cleft lip and palate	Cleft palate	Dead	Percentage cleft palate
1	*Tw*+ × *Tw*+	7	53	15	..	1	21·7
2*a*	*Tw*+ × ++	14	83	..	..	6	0·0
2*b*	*Tw*+ × *Tw*+	26	160	10	10	5	10·8
3	*Tw*+ × *Tw*+	7	29	5	4	..	23·7

To find the missing homozygotes *Tw*+ females were mated to *Tw*+ males and killed at 14–17 days' gestation for examination of the foetuses (Table 2, series 1). Roughly one-quarter (15 out of 69) of the young showed harelip, and these animals were taken to be the missing homozygotes. Later, a second series of embryo counts was made to check that no harelip young occurred after matings of *Tw*+ × ++. This was in fact confirmed (Table 2, series 2*a*). Simultaneously another series of *Tw*+ × *Tw*+ matings was made. For this series twirlers from a different outcross from those in the first were used, and females were opened at 18½ days' gestation. Among the progeny of the *Tw*+ × *Tw*+ matings of this series (Table 2, series 2*b*) there were some foetuses which had cleft palate without cleft lip as well as those with cleft lip and cleft palate. Even so, however, the proportion of abnormals fell short of the expected 25 per cent. In yet another series, in the course of linkage tests, twirler females mated to twirler males were allowed to litter and the young were examined at birth for harelip and cleft palate. In this group (Table 2, series 3) the proportion of putative homozygotes was again roughly a quarter. The explanation of the shortage in the second series is not clear; as only five dead embryos were found it cannot be supposed that the missing abnormal foetuses had died. Incomplete penetrance of the homozygous phenotype is a possibility; in such a case tests of the twirler young of intercrosses should have revealed some living homozygotes. Nine of the 24 young tested came from this stock, and none gave any suspicion that they were other than heterozygotes. Thus, while it may be accepted that the animals with harelip and cleft palate represent the twirler homozygotes, it is not known why the expected proportion is not always found.

Linkage tests

The results of linkage tests with *Tw* are shown in Table 3. In the cross with dreher, *dr*, twirlers could be distinguished from drehers by observing the deafness of the latter, but among the drehers the twirler drehers were not distinguished. Similarly, in the cross with jerker, *je*, twirlers could be distinguished from jerkers by their ability to hear, but in this case twirlers could also be distinguished within the jerker class by observing the morphology in preparations of the ear.

TABLE 3

Linkage tests with twirler

Mutant m/M	*Mating type*	*Tw+*	*++*	*Tw m*	*+m*	*Total*	*R.F.*	χ^2	*S.E.*
a	RB	22	29	21	29	101	50·5	0·010	5·0
ax	CM	112	64	..	47	223	0·0	53·40	..
	CI	15	..	..	6	21	0·0	..	..
b	RB	22	25	20	34	101	55·4	1·198	5·0
c^{ch}	RB	30	48	36	38	152	44·7	1·684	4·1
d	RB	24	34	22	25	105	46·7	0·467	4·9
dr	CB	34	43	82		159	55·8	1·052	5·7
fz	CB	34	39	23	28	124	50·0	0·0	4·5
je	CB	5	3	6	5	19	47·4	0·053	11·5
	CM	14	23	6	4	47	66·0	1·597	12·6
ln	CB	24	29	33	38	124	50·0	0·0	4·5
ru	CB	24	22	14	26	86	41·9	2·279	5·4
s	RB	23	33	21	26	103	47·6	0·243	4·9
wa−2	CB	16	20	18	22	76	50·0	0·0	5·7
		TwM	*+M*	*Tw+*	*++*				
Ca	RB	23	27	28	28	106	48·1	0·151	4·9
Mi^{wh}	RB	26	22	22	31	101	56·4	1·673	5·0
Ra	RB	26	32	26	35	119	51·3	0·076	4·6
Re	RB	24	26	28	28	106	49·1	0·038	4·9
Sd	RB	17	29	15	24	85	48·2	0·106	5·4
T	RB	25	25	20	20	90	50·0	0·0	5·3
W^v	RB	21	28	30	28	107	45·8	0·757	4·8

C = coupling; R = repulsion; B = backcross; I = intercross; M = backcross for *Tw*, intercross for *m*.

In the tests of *Tw* with *ax* the linkage χ^2 is highly significant and no *Tw+ axax* young were found. The greater part of the data, however, are from matings of the type *Tw+ +ax* × *++ +ax* (CM) and from this type of mating the *Tw+ axax* young would be the only known recombinant type. It is possible that these twirler ataxics would either die or not be distinguishable from either twirler or ataxia alone, thereby giving a false impression of linkage. The hypothesis of a true linkage is favoured, however, on two grounds. Firstly, the proportions of the three types of young found in the progeny of these mixed crosses are in agreement with those expected on the hypothesis of linkage and differ from those expected on other hypotheses. Secondly, among the offspring of matings of the

type *Tw*+ +*ax* × *Tw*+ +*ax* not only twirler ataxic but also normal animals would be recombinants and, in agreement with the hypothesis of linkage, none have so far been found among the small number of young examined.

It may therefore be accepted that twirler is closely linked to ataxia. Ataxia has previously been shown to segregate independently of marker genes in most of the known linkage groups (Lyon, 1955). Hence *Tw* and *ax* belong to a new group, for which the number XV is proposed. As no recombinants have yet been found the observed recombination between the two genes is 0 per cent.

Possible allelomorphism of twirler with similar mutants

There are many known mutants of the mouse which produce 'waltzing' behaviour similar to that of twirler. For most of these, however, the discovery of the linkage relations of *Tw* rules out the possibility of it being a new allelomorph of an already known gene. As Table 3 shows, direct tests of twirler with dreher and jerker gave no evidence of allelomorphism or linkage. The six ear mutants listed below are known to be linked to the genes shown, with which *Tw* shows no sign of linkage.

		Linked to
fidget	*fi*	*Sd*
kreisler	*kr*	*a*
pallid	*pa*	*a*
pirouette	*pi*	W^v
shaker-1	*sh*-1	c^{ch}
shaker-2	*sh*-2	*wa*−2

Allelomorphism with waltzer, *v*, or varitint-waddler, *Va*, can similarly be ruled out as these two mutants show no linkage with *ax* (Lyon, 1955), with which *Tw* is closely linked. Twirler has not yet been tested for allelomorphism with zigzag, *Zg* (Lyon, unpublished).

DISCUSSION

The linkage of twirler with ataxia means that there are now sixteen known linkage groups in the mouse, autosomal linkage groups I–XV and the sex chromosomal group XX. As group XV is so short, however, no known recombinants having yet been found, it could represent a remote part of an already known group; a point which will only be decided by future linkage tests with new mutants. Since the mouse has twenty pairs of chromosomes there now remain at least four chromosomes not marked by linkage groups, or more than four if some of the known groups are not really independent.

In its morphological effects twirler is interesting as being an addition to the list of mouse mutants which affect the inner ear. Grüneberg (1956), in reviewing the knowledge of these mutants, divides the majority of them into two classes: those in which the labyrinth shows morphological abnormalities and those in which degenerative changes occur in a morphologically normal labyrinth. Six or more mutants are now known in each group, in the first of which twirler clearly belongs. The mutants of this group now provide valuable material for

study of both the physiology and the embryology of the ear. In Table 4 the defects found in each of these mutants are tabulated according to severity. Twirler occupies an intermediate position. Less affected are pallid, in which the only ear defect is absence of otoliths, and zigzag, in which the otoliths are normal but the horizontal canals are absent (Lyon, unpublished). The mutants twirler, dreher, and kreisler appear to form a series of increasing grade of defect, and embryological studies aimed at finding some common origin for their abnormalities would be valuable.

TABLE 4

The defects of some mutants with morphological effects on the inner ear

Ear defect				*Behaviour*		
Otoliths	*Horizontal canal*	*Vertical canals*	*Mutant*	*Position response*	*Horizontal movement*	*Vertical movement*
A	N	N	Pallid	A	N	N
N	A	N	Zigzag	N	A	N
N	A	A	Fidget	N	A	N
N/A	A	N/A	Twirler	N/A	A	N/A
N/A	A	A	Dreher	N/A	A	A
A	A	A	Kreisler	A	A	A

A = abnormal; N = normal.

Nothing is yet known of the basis of the pleiotropy of twirler. In heterozygotes there is the combination of obesity with ear defect, and in homozygotes cleft palate, cleft lip, and ear defect are combined. Cleft lip and palate are well known in the inbred strain A (Reed, 1936). More recently cleft palate without cleft lip has been produced in mouse foetuses by treatment of pregnant females with cortisone (Fraser & Fainstat, 1951). It has also been reported briefly as occurring in mice homozygous for the mutant *ur* (Gluecksohn-Waelsch & Kamell, 1955). No link with ear defect is yet apparent. Hereditary obesity of the mouse has also been reported more than once. The obesity of mice carrying the mutant yellow, A^y, has been known from the early days of mouse genetics. More recently the mutant obese, *ob*, has been found (Ingalls, Dickie, & Snell, 1950), and obesity of unknown genetic cause has been reported in the NZO strain (Bielschowsky & Bielschowsky, 1956). In both these types of obesity there is hyperglycaemia, although the underlying hormonal or metabolic causes are thought to be different. The *obob* mice are sterile, as are obese twirlers. Again no link with ear defect is obvious.

SUMMARY

Twirler, *Tw*, is a new spontaneous mutant of the house mouse which, when heterozygous, causes abnormal behaviour, including circling, head-shaking, and absence of postural reflexes, which can be attributed to morphological defects

of the inner ear. Homozygotes have cleft lip and palate in addition to ear abnormality, and die soon after birth. The ear defects of heterozygotes include absence of otoliths and reduction and malformation of canals. The animals are not deaf and the cochlea appears normal. Twirler is closely linked to ataxia, *ax*, this being the first linkage of a new linkage group, number XV.

ACKNOWLEDGEMENTS

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EXPLANATION OF PLATE

Fig. A. Transverse section of ear of twirler heterozygote showing the utriculus and horizontal ampulla. In the utriculus the otolith granules are lacking. In the ampulla the crista is malformed, being infolded to form a pit rather than a ridge. ×90.

Fig. B. Transverse section of the utriculus of a normal mouse showing otolith granules. ×90.

Fig. C. Section of the horizontal ampulla of a normal mouse showing the normal shape of the crista. ×90.

Fig. D. Higher magnification of the abnormal crista shown in Fig. A. ×290.

Fig. E. Higher magnification of the utricular macula shown in Fig. A. ×290.

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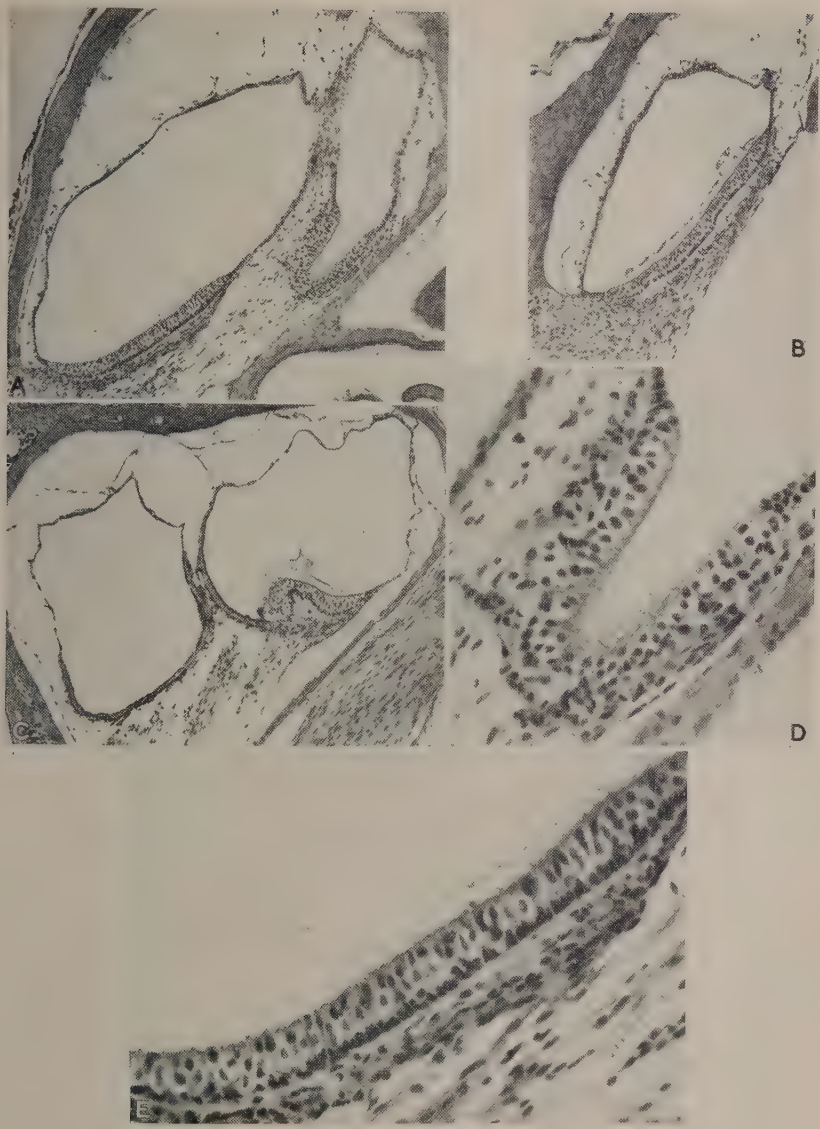
A
B
D

Étude autoradiographique de l'incorporation de $^{14}CO_2$ dans des gastrulas d'Axolotl

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INTRODUCTION

De nombreux précurseurs radioactifs des protéines et des acides nucléiques ont été utilisés par divers auteurs afin de suivre le métabolisme au cours du développement embryonnaire des Batraciens. Les résultats intéressants qui ont été déjà obtenus par les méthodes biochimiques et par la technique autoradiographique engagent à poursuivre dans cette voie.

Friedberg & Eakin (1949), utilisant la glycine marquée au C^{14}, ont remarqué la faible pénétrabilité pour cet acide aminé, à partir de la gastrula. Afin d'augmenter la vitesse d'incorporation, ils se sont vus obligés de travailler sur des fragments d'embryons. Afin d'éviter cet inconvénient et l'emploi de temps d'incubation assez longs — ce qui risque de diminuer la précision des résultats — deux auteurs ont utilisé le C^{14} sous forme de bicarbonate. C'est ainsi que Cohen (1954) a montré que le $^{14}CO_2$ s'incorpore dans les acides nucléiques et les protéines, après être entré dans le cycle de Krebs. Un travail simultané de Flickinger (1954) a donné des résultats semblables; mais cet auteur s'est attaché, en outre, à l'étude de l'incorporation régionale et il a montré qu'il existe des différences marquées entre les différentes régions.

En ce qui concerne l'autoradiographie, signalons surtout les travaux de Ficq (1954), de Sirlin & Waddington (1954) et de Sirlin (1955). Ficq (1954), utilisant la technique des émulsions nucléaires, a analysé l'induction neurale en greffant des organisateurs marqués au préalable soit au glycocolle, soit à l'acide orotique. Elle a observé, entre autres, que l'activité est très nettement localisée dans les noyaux et que les régions de morphogenèse active sont celles qui se marquent le plus. Sirlin & Waddington (1954) et Sirlin (1955), utilisant la méthode de stripping de Doniach & Pelc (1950), ont suivi le sort de la glycine et de la méthionine dans des embryons de Triton. Ils ont également observé une activité nucléaire intense; pour ces auteurs, l'activité serait prédominante dans le mésoblaste. Notons toutefois, que tous ces résultats ont été obtenus après des temps d'incubation très longs, en raison de la lente incorporation de ces acides aminés.

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C'est pourquoi nous avons utilisé le $^{14}CO_2$ qui diffuse rapidement à travers la membrane vitelline et le coat des embryons; nous avons alors suivi, par autoradiographie, le sort de ce précurseur dans des gastrulas d'Axolotl, en examinant l'incorporation dans les différents territoires et en comparant les noyaux au cytoplasme.

MÉTHODES

Les œufs d'Axolotl obtenus par ponte spontanée étaient dégangués et lavés dans une solution saline ayant la composition suivante: NaCl, 350 mg.; KCl, 5 mg.; $CaCl_2$, 10 mg.; H_2O, 1.000 c.c. 2 c.c. de la solution saline additionnée de bicarbonate radioactif (concentration finale 4 μc./c.c. soit 56 γ/c.c.) étaient versés dans un pèse-filtre. Dix à vingt œufs étaient traités, dans un pèse-filtre, pendant une durée de 5 heures. Après ce laps de temps, les embryons étaient lavés et fixés à l'alcool-acétique à froid (acide acétique à 5 pour cent dans l'alcool absolu refroidi à $-8°$) pendant 4 heures. Après un séjour de 12 heures dans de l'alcool absolu, également à $-8°$, les œufs étaient placés dans du toluol préalablement refroidi; ils étaient enfin ramenés à la température ordinaire et enrobés dans la paraffine. Les coupes de 10 microns étaient déposées sur des lames recouvertes au préalable d'un film de gélatine alunée, permettant l'application de l'émulsion. Certaines lames ont été traitées à la ribonucléase pendant 1 à 2 heures à 38° (conc. 0,4 à 1 mg./c.c.) afin d'éliminer l'activité de l'ARN. Un autre lot de préparations a été soumis à l'action successive de la ribonucléase et de la désoxyribonucléase (2 heures dans une solution à 0,1 mg./c.c. de NaCl 9‰ additionnée de Mg^{++} M/300). Après un lavage soigneux des lames hydrolysées et séchage, l'émulsion nucléaire était appliquée suivant la technique décrite par Ficq (1955). Les durées d'exposition étaient de 5 jours. Après révélation et fixation, toujours suivant la technique de Ficq, les préparations ont été colorées au mélange vert de méthyl-pyronine pendant 10 minutes environ, lavées à l'eau courante et montées à la glycérine.

RÉSULTATS

Voice les résultats obtenus pour l'incorporation totale du CO_2:

Nous avons étudié quatre régions de jeune gastrula (cf. fig. 1). Le tableau 1 donne les résultats. C'est le neuroblaste qui présente la plus forte activité; l'ectoblaste est un peu plus actif que le chordomésoblaste, tandis que l'entoblaste est très peu actif. D'une façon générale, l'activité est élevée dans les noyaux.

Les territoires de la gastrula avancée où les traces ont été dénombrées sont indiqués sur la fig. 2. Les résultats sont résumés dans le tableau 2. L'activité se distribue de la même façon ($A > B > C > D$) que dans la gastrula jeune.

Enfin, nous avons déterminé l'activité par unité de surface pour le cytoplasme et le noyau. Les tableaux 3 et 4 résument les résultats obtenus aux deux stades étudiés. Nos observations montrent que, dès la jeune gastrula, des gradients

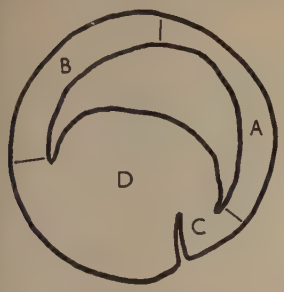

FIG. 1. Jeune gastrula. Territoires étudiés: A, neuroblaste présomptif; B, ectoblaste; C, région correspondant à l'organisateur; D, portion entoblastique.

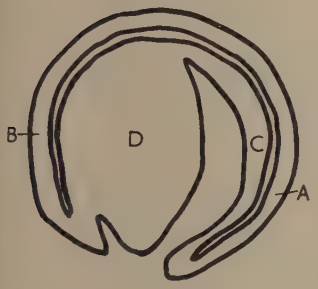

FIG. 2. Gastrula avancée. Territoires étudiés: A, neuroblaste; B, ectoblaste ventral; C, chordomésoblaste; D, portion entoblastique.

TABLEAU 1

Résultats relatifs à la jeune gastrula. Nombre moyen de traces par unité de surface des quatre territoires étudiés (cf. fig. 1)

Régions	*Nombre de traces/156 μ^2*	*Nombre total de traces/ Nombre total de surfaces*
A. Neuroblaste . . .	3,12	572/183
B. Ectoblaste . . .	1,83	737/402
C. Région de l'organisateur	1,52	711/405
D. Portion entoblastique .	0,50	166/331

Dans la colonne de droite, figurent le nombre total de traces et le nombre total de surfaces dénombrées.

TABLEAU 2

Résultats relatifs à la gastrula avancée. Nombre moyen de traces par unité de surface des quatre territoires étudiés (cf. fig. 2)

Régions	*Nombre de traces/156 μ^2*	*Nombre total de traces/ Nombre total de surfaces*
A. Neuroblaste . . .	4,14	1556/372
B. Ectoblaste ventral . .	2,91	993/341
C. Chordomésoblaste. .	1,00	546/541
D. Portion entoblastique .	0,55	131/238

TABLEAU 3

Jeune gastrula. Nombre moyen de traces par unité de surface

Régions	*Noyau*	*Cytoplasme*
A	4,93 (291/59)	1,79 (172/96)
B	4,84 (659/136)	1,20 (34/11)
C	4,65 (652/140)	0,70 (145/205)
D	4,16 (129/31)	0,27 (116/420)

Entre parenthèses, figurent le nombre total de traces et le nombre total de surfaces dénombrées.

TABLEAU 4

Gastrula avancée. Nombre moyen de traces par unité de surface

Régions	*Noyau*	*Cytoplasme*
A	7,44 (849/114)	2,45 (59/24)
B	5,23 (361/69)	1,46 (49/33)
C	4,96 (164/33)	0,83 (150/179)
D	2,30 (23/10)	0,48 (64/131)

Entre parenthèses, figurent le nombre total de traces et le nombre total de surfaces dénombrées.

d'activité sont très reconnaissables dans le cytoplasme, sans qu'il y ait de grandes différences pour les noyaux; au stade gastrula avancée par contre, les noyaux présentent des gradients d'activité très nets, parallèles à ceux qu'on trouve dans le cytoplasme aux deux stades étudiés.

En ce qui concerne la distribution de l'activité parmi les différentes substances marquées, nous ne pouvons faire état que de quelques résultats se rapportant à la gastrula moyenne (fig. 3). Trois embryons seulement ont été observés. Les tableaux 5 et 6 rendent compte de ces résultats.

TABLEAU 5

Résultats relatifs à la gastrula moyenne

Régions:	A	B	C	D
1. Témoin	3,71 (2692/725)	2,42 (2169/893)	0,60 (618/1026)	0,37 (397/1063)
2. Après RNase . . .	0,91 (579/630)	0,69 (328/474)	0,29 (122/410)	0,18 (93/506)
3. Après RNase et DNase .	0,30 (178/591)	0,26 (149/563)	0,29 (147/499)	0,19 (82/427)

Ce tableau indique les moyennes obtenues: (1) pour l'activité totale (témoin); (2) après hydrolyse à la ribonucléase; (3) après hydrolyse successive à la ribonucléase et à la désoxyribonucléase.

Entre parenthèses, figurent le nombre total de traces et le nombre total de surfaces dénombrées.

TABLEAU 6

Distribution de la radioactivité parmi les différentes substances marquées

Régions:	A	B	C	D
1. ARN . . .	2,80	1,73	0,31	0,19
2. ADN . . .	0,61	0,43	—	—
3. Protéines . .	0,30	0,26	0,29	0,19

Ces moyennes ont été obtenues:

1. par différence entre les moyennes obtenues pour l'activité totale (ARN + ADN + protéines) et celles obtenues après élimination de l'ARN;
2. par différence entre les moyennes obtenues après l'élimination de l'ARN et celles obtenues après élimination de l'ARN + ADN;
3. par dénombrement des traces résiduelles après élimination de l'ARN et de l'ADN.

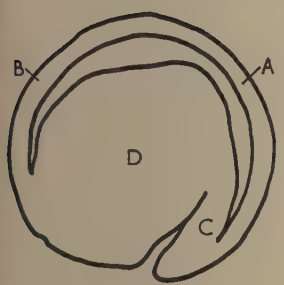

FIG. 3. Gastrula moyenne. Territoires étudiés: A, neuroblaste présomptif; B, ectoblaste; C, organisateur; D, entoblaste.

DISCUSSION

En ce qui concerne l'incorporation totale du $^{14}CO_2$, nos observations autoradiographiques sont en bon accord avec les résultats de Flickinger (1954) qui a travaillé avec un compteur: il a observé des gradients d'activité, analogues à ceux que nous venons de décrire, tant en ce qui concerne les protéines que les

acides nucléiques. Toutefois, nos premiers résultats n'indiquent de gradients que pour l'ARN au début de la gastrulation.

Ces gradients d'activité sont superposables aux gradients ribonucléiques, animal-végétatif et dorso-ventral, qui ont été observés cytochimiquement par Brachet (1944, 1952) et dont l'existence a été vérifiée par le dosage direct de l'ARN (Steinert, 1954; Takata, 1953).

Comme l'ont signalé Ficq (1954) et Sirlin (1955), la radioactivité est surtout concentrée dans les noyaux. Dès lors, il n'est pas étonnant de trouver une activité globale plus grande dans la partie animale, où les noyaux sont plus abondants. Mais l'examen de la distribution de l'activité cytoplasmique dans la jeune gastrula montre un parallélisme entre l'augmentation du nombre de traces et la diminution de la taille des plaquettes vitellines. Les différences métaboliques qui existent, au stade jeune gastrula, entre le neuroblaste et l'ectoblaste présomptifs étaient cependant inattendues: en effet, de nombreuses expériences (d'explantation, de transplantation, d'exogastrulation, etc.) ont montré que, en l'absence d'induction, ni la plaque médullaire présomptive, ni l'ectoderme présomptif d'une jeune gastrula ne sont capables de former du tissu nerveux.

Par contre, on peut mettre en évidence expérimentalement des différences entre le mésoblaste dorsal et ventral à ce stade. Nous savons toutefois par des expériences de Yamada (1950) et de Kawakami & Okano (1955) que la détermination est encore labile à ce moment. C'est à ce même stade que l'organisateur transforme l'ectoderme compétent en système nerveux, que différents agents chimiques peuvent provoquer la neuralisation, que la précytolyse, comme l'a montré Holtfreter (1947), suffit à provoquer la neuralisation spontanée de l'ectoblaste.

Au moment où les territoires cytoplasmiques commencent à se différencier, dès la jeune gastrula, les noyaux présentent encore, en ce qui concerne l'incorporation du $^{14}CO_2$, des radioactivités très voisines. Par contre, au stade gastrula avancée, les noyaux des divers territoires semblent s'être individualisés. Une pareille constatation concorde avec les résultats obtenus par Briggs et King qui ont transplanté des noyaux de jeune gastrula (Briggs & King, 1952) et de gastrula avancée (King & Briggs, 1955) dans des œufs vierges préalablement énucléés. Ils ont conclu de leurs expériences que les noyaux n'ont pas encore subi de changements irréversibles et qu'ils ont conservé toutes leurs potentialités dans la jeune gastrula et que c'est au cours de la gastrulation qu'ils se différencient. Il serait, croyons-nous, intéressant d'étendre ces résultats au cas des embryons dont le développement a été altéré expérimentalement (gastrulas centrifugées ou lithinées, hybrides létaux, etc.) afin de préciser la nature des échanges entre le noyau et le cytoplasme au cours de la morphogenèse.

RÉSUMÉ

L'incorporation du $^{14}CO_2$ a été suivie, dans différents territoires de la gastrula

d'Axolotl, par la méthode autoradiographique. Dès la jeune gastrula, on observe des gradients d'activité animal-végétatif et dorso-ventral. Ces différences affectent surtout le cytoplasme; les noyaux sont très actifs, mais ils présentent des radioactivités très voisines. On retrouve les mêmes différences au stade gastrula avancée. Mais à ce moment, les noyaux présentent des gradients d'activité parallèles à ceux qu'on trouve dans le cytoplasme.

SUMMARY

Regional incorporation of $^{14}CO_2$ into Amphibian gastrulae has been followed by autoradiography. Differences in uptake of the tracer in different parts appear already in the early gastrula. These differences only affect the cytoplasm while the nuclei, heavily labelled, show very similar radioactivities. In the late gastrula, however, the nuclei show the same gradients of activity as the cytoplasm.

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Genetical Studies on the Skeleton of the Mouse[1]

XXII. The Development of Danforth's Short-tail

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WITH THREE PLATES

INTRODUCTION

THE semi-dominant gene for Danforth's short-tail in the mouse (symbol *Sd*; linkage group V) was first described by Dunn, Gluecksohn-Schoenheimer, & Bryson (1940). The most conspicuous abnormality of *Sd*/+ heterozygotes is a shortening of the tail the extent of which varies with the genetic background (Dunn, 1942; Fisher & Holt, 1944; Dunn & Gluecksohn-Schoenheimer, 1945). Reduction or absence of kidneys is common on some genetic backgrounds, but rare or absent on others (Gluecksohn-Schoenheimer, 1943). Reduction or absence of the dens epistrophei (odontoid process of the axis) with formation of an anomalous articulation between atlas and epistropheus (axis) was later described by Theiler (1951 *a*, *b*; 1952; 1954) and by Grüneberg (1953). The reduction of the dens epistrophei is part and parcel of a general reduction of the vertebral bodies which is most marked in the cervical region, but which can be traced throughout the whole length of the axial skeleton. *Sd*/*Sd* homozygotes (Gluecksohn-Schoenheimer, 1943) are either completely tailless or have a tail filament only; in the absence of rectum and anus there is a persistent cloaca; the bladder is reduced or absent, as is the urethra and the genital papilla; the kidneys are usually completely lacking, and the *Sd*/*Sd* homozygotes almost invariably die within 24 hours after birth. It has been claimed that on certain genetic backgrounds, the *Sd*/*Sd* homozygotes may have enough functioning kidney tissue to permit survival for a longer period (Fisher & Holt, 1944), but the evidence for this assertion has been criticized by Dunn & Gluecksohn-Schoenheimer (1945).

Both in *Sd*/+ and in *Sd*/*Sd* embryos, massive cell degenerations (with or without haemorrhagic lesions) occur in those parts of the embryonic tail which

[1] The first twenty-one papers of this series, by the present author and by various other members and guests of this research group, have appeared in the *Journal of Genetics*, vols. **50–55,** 1950–7.

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will subsequently regress (Gluecksohn-Schoenheimer, 1945). There is also a more or less complete breakdown of the notochord throughout its length which Gluecksohn-Schoenheimer regarded as secondary. The cause for the regressive processes in the tail was not discovered. Nor did Gluecksohn-Schoenheimer find a connecting link between the abnormalities of the axial skeleton and those of the urogenital system and the persistence of the cloaca.

The recent discovery of the ventral ectodermal ridge of the tail (Grüneberg, 1956) suggested a re-investigation of the embryology of *Sd*/+ and *Sd*/*Sd* mice. The ventral ectodermal ridge of the tail is a transitory thickening of the ectoderm which is somewhat similar to the apical ectodermal ridge of the limb-buds in avian and mammalian embryos, and it is believed that it may have a comparable stimulatory function on the outgrowth of the tail-bud. This is strongly suggested by the fact that in vestigial-tail (*vt*/*vt*) mice, early regression of the embryonic tail is accompanied by a considerable reduction of the ventral ectodermal ridge (Grüneberg, 1957*a*). As the ventral ectodermal ridge originates at the cloacal membrane whence it spreads in a distal direction, it seemed conceivable that the connecting link in the *Sd*-syndrome might be some abnormality of the cloaca which indirectly, through the ventral ectodermal ridge, interferes with tail growth (Grüneberg, 1957*b*). As will be discussed below, this has not proved to be the case. On the other hand, facts have come to light which strongly suggest a different unitary explanation of the *Sd*-syndrome.

MATERIALS AND METHODS

Sd/+ males from a mixed stock were mated to +/+ F_1 females from a cross between the inbred strains C57BL/Gr and CBA/Gr (Table 1). With the exception of litter 1, +/+ and *Sd*/+ embryos could not be distinguished by external features and were thus classified from sections; litters 2–12 included 28 +/+ and 35 *Sd*/+ embryos respectively, in reasonable agreement with a 1 : 1 expectation. The *Sd*/+ embryos, at any given stage of development, were in agreement with each other in all essential features. They differed from each other to some extent in the degree of abnormality, and these differences between known *Sd*/+ heterozygotes served as a base line for the identification of the *Sd*/*Sd* homozygotes derived from *Sd*/+ × *Sd*/+ matings (Table 2). With the exception of litter 13, the +/+, *Sd*/+ and *Sd*/*Sd* embryos could not be distinguished from each other by external inspection; as classified from sectioned material, litters 14–26 included 23 +/+, 40 *Sd*/+ and 18 *Sd*/*Sd* embryos, in good agreement with a 1 : 2 : 1 expectation. Altogether, 151 embryos ranging in age from 9 to $12\frac{1}{2}$ days have been serially sectioned. The material was fixed in Bouin's fluid and embedded by Peterfi's method. Sections were cut at 7·5 μ except in the case of the large embryos of litters 1 and 13 which were sectioned at 12·5 μ. The sections were stained with haematoxylin and eosin. Great care was taken to obtain, as nearly as possible, transverse sections through the tail, the tail-bud, or

the posterior end of the body; the relevant features are easily recognized in transverse sections, but in other orientations interpretation is often difficult or impossible. Projection drawings at magnification ×250 were made of every

TABLE 1

Serially sectioned embryos from Sd/+ × +/+ matings

Litter	Nominal age	Crown-rump length (mm.) +/+	Crown-rump length (mm.) Sd/+	Mean C.R.L.
1	12½	8·5 8·5	8·2 8·1	8·3
2	10	3·8 4·2	4·3 4·3 3·8	4·1
3	10	4·5 3·7 3·7 4·4	3·4 4·0 3·8 4·2	4·0
4	10		4·0 3·4 3·8 3·6 3·9 3·2	3·7
5	9¾	3·1 3·5		3·3
6	9½	3·0 2·7 2·4 2·5	3·1 2·8	2·8
7	9½	2·7 2·3 2·9 2·8	2·8 2·6	2·7
8	9½		3·1 2·9 2·4 2·8 2·4 2·5	2·7
9	9½	2·8 2·4 1·9 2·2 2·2	2·6 3·1 2·6	2·5
10	9½	2·5 2·7 2·5	2·7 2·7 2·3 1·9 2·0	2·4
11	9	2·6 2·5 2·0 1·9		2·3
12	9		1·8 2·0 1·6 2·1	1·9

TABLE 2

Serially sectioned embryos from Sd/+ × Sd/+ matings

Litter	Nominal age	Crown-rump length (mm.) +/+	Crown-rump length (mm.) Sd/+	Crown-rump length (mm.) Sd/Sd	Mean C.R.L.
13	12½	8·3	7·9	7·1	7·8
14	10½	4·9 4·6	5·0 4·7 5·5 4·7 4·7	4·7 3·6	4·7
15	10½	4·1 5·2 5·0	4·8 4·4 4·7 4·3 4·6		4·6
16	10	4·2 4·2	4·5 4·0	4·1 3·8 3·9	4·1
17	10	4·0	4·2 4·0 4·0 4·2	3·7	4·0
18	10	3·7 3·7 3·6	3·8 3·7 4·0 3·6	3·8 3·5 3·7	3·7
19	9¾	3·0 3·5	3·4 3·0 3·0		3·2
20	9¾	3·4 3·3	3·4 3·5 2·6 3·3 3·1 2·9		3·2
21	9½	2·9 3·0	3·3	3·1 3·0	3·1
22	9½	2·7 2·5 2·0	3·0	3·1	2·7
23	9½	2·6	2·7 2·5 2·5	2·5	2·6
24	9½	2·8	2·9 2·1 2·4		2·6
25	9	2·4	1·9 1·9	2·3 2·5 2·0	2·2
26	9		2·1	2·1 2·2	2·1

10th section of the relevant regions of each embryo. The simultaneous comparisons which can be made between sheets of such drawings greatly facilitate the detection of differences which may be missed by other methods.

The ages of the embryos as given in Tables 1 and 2 are purely nominal. There may be considerable differences in size (and in stage of development) between members of the same litter. Moreover, the time taken by a litter to reach a given stage of development may differ considerably from stock to stock; these varia-

tions are probably largely a function of the maternal physiology (fitness). A set of embryos described some time ago (Grüneberg, 1943) is in fair agreement with a more recent set of Otis & Brent (1954). However, in vigorous stocks, developmental age may be ahead of the 'standard' by as much as 24 hours, and a similar delay may be found in inbred or otherwise feeble strains. In comparing the results of different authors, the chronological age of embryos is thus virtually useless. The mean crown-rump length of a litter is more reliable. The best method is to describe the embryos in terms of a standard set of developmental stages or 'horizons'. This will have to be borne in mind in comparing present data with those of Gluecksohn-Schoenheimer (1945).

THE GENERAL DEVELOPMENT OF *Sd*/+ AND *Sd*/*Sd* EMBRYOS

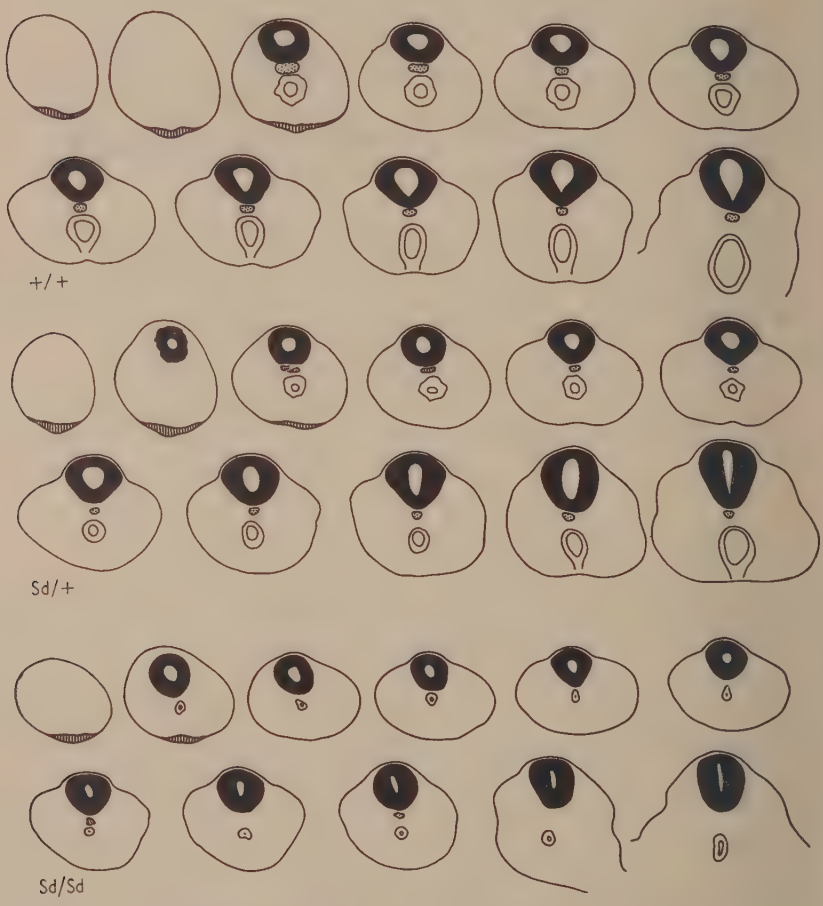
The development of the major features of *Sd*/+ and *Sd*/*Sd* embryos will be described by reference to four trios of embryos which can be regarded as representative of the material as a whole, as follows:

Text-figure	*Litter*	*Mean C.R.L. (mm.)*	*Nominal age (days)*
1	18	3·7	10
2	21	3·1	9½
3	23	2·6	9½
4	25	2·2	9

At the age of 10 days the distinction between +/+, *Sd*/+, and *Sd*/*Sd* embryos presents no difficulties (Text-fig. 1). *Sd*/*Sd* embryos can be distinguished from +/+ normals at this stage by three major features. (1) The notochord, in its caudal parts, is ill-defined, and over considerable stretches cannot be identified as a separate structure. (2) The tail-gut is very small and often lacks a lumen; it may be interrupted in places, and not infrequently it is so intimately fused with cells of apparent notochordal origin that the two cannot be separated from each other. The reduction of the tail-gut continues anteriorly in the cloaca which is greatly reduced in size. (3) Cell degenerations (pyknoses) are present in various axial structures. They are most marked in the centre of the somites and here are particularly obvious at the level of the cloaca. Pyknotic cell nuclei also occur in the neural tube, particularly in its dorsal region in a position corresponding approximately to the (now closed) posterior neuropore; they thus extend beyond the region yet reached by the formation of somites. (All the characteristics by means of which *Sd*/*Sd* embryos can be identified later on, such as the presence of a cloaca, absence of bladder, urethra and genital papilla, and absence of a metanephros are, at this early stage, of course normal embryonic features.)

The 10-day *Sd*/+ embryos are about intermediate between the +/+ and the *Sd*/*Sd* homozygotes. The notochord of the tail region is a continuous structure fairly well separated from its neighbours; however, it differs from a normal noto-

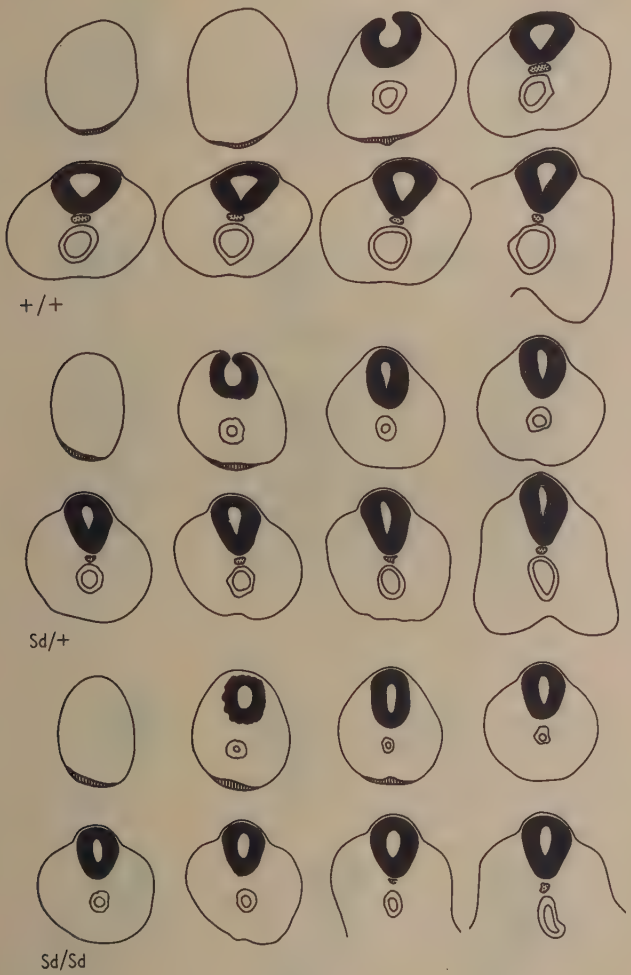
chord by its smaller calibre, by a tendency to an irregular outline in cross-section, and by a generally less solid structure; it is indeed quite easy to distinguish from a normal notochord. The tail-gut is, on an average, clearly smaller than in



TEXT-FIG 1. Projection drawings of transverse sections through the tails of mouse embryos. In each case the 10th, 20th, 30th, &c., section, starting from the tail tip, has been drawn. Neural tube (or plate) in black, notochord stippled, tail-gut white and ventral ectodermal ridge hatched. First two rows $+/+$, next two rows $Sd/+$, and last two rows Sd/Sd embryo (C.R.L. 3·6 mm., 3·7 mm., and 3·8 mm. respectively; litter 18; nominal age 10 days). Sections 7·5 μ thick. Drawn at magnification $\times 250$; final magnification $\times 62{\cdot}5$.

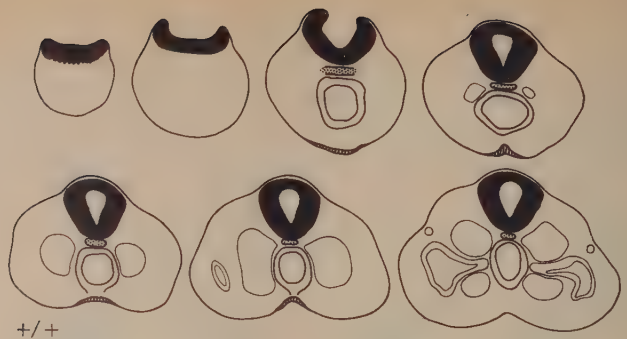
normal embryos, but the distributions overlap to some extent. It is much larger than the tail-gut of Sd/Sd embryos, and the cloaca is of about normal dimensions. Some $Sd/+$ embryos show slight signs of cell pyknosis; in others there is

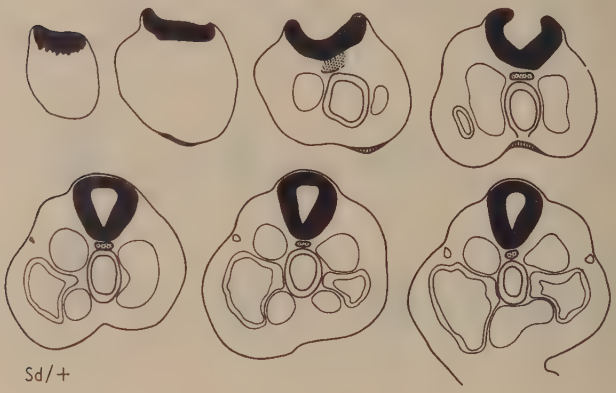
none (other than the pyknosis occurring in normal development; see Glücksmann, 1951).

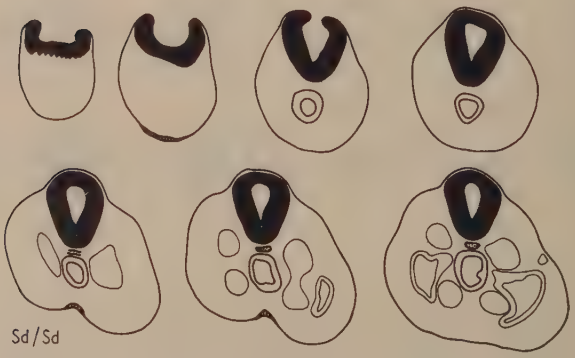


TEXT-FIG. 2. Projection drawings of transverse sections through the tails of mouse embryos. First two rows + / +, next two rows *Sd* / +, and last two rows *Sd* / *Sd* embryo (C.R.L. 2·9 mm., 3·3 mm., and 3·1 mm. respectively; litter 21; nominal age 9½ days). All conventions and magnifications as in Text-fig. 1.

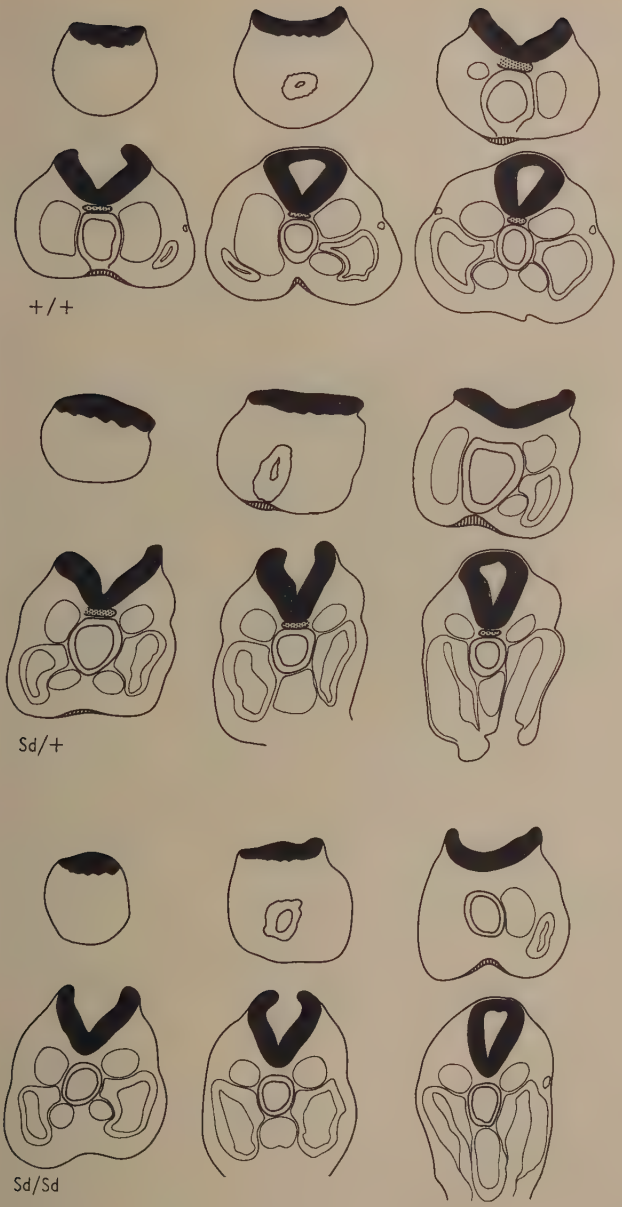
At a somewhat earlier stage (9½ days, Text-fig. 2), the three genotypes can be distinguished by the same criteria, except that there is little or no cell pyknosis in *Sd* / *Sd* embryos at this stage; where it occurs, it is present mainly in the dorsal aspect of the neural tube. Otherwise, the most distal parts of the *Sd* / *Sd*







TEXT-FIG. 3. Projection drawings of transverse sections through the tail-bud and the posterior end of the trunk region of mouse embryos. The first two rows $+/+$, the next two rows *Sd*/+, and the last two rows *Sd*/*Sd* embryo (C.R.L. 2·6 mm., 2·5 mm., and 2·5 mm. respectively; litter 23; nominal age 9½ days). In these drawings and those of Text-fig. 4 the dorsal aortae, the roots of the umbilical artery, and the common umbilical artery are indicated by single contours; the coelom is shown by double contours; and the Wolffian duct, immediately underneath the epidermis and still without a lumen at this level, is also shown, e.g. in the 70th section of the $+/+$ embryo.



TEXT-FIG. 4. Projection drawings of transverse sections through the posterior end of the trunk region of mouse embryos. First two rows $+/+$, next two rows $Sd/+$, and last two rows Sd/Sd embryo (C.R.L. 2·4 mm., 1·9 mm., and 2·0 mm. respectively; litter 25; nominal age 9 days).

notochord (i.e. those last formed) are reduced and diffuse while more anteriorly the notochord is a clearly recognizable (though by no means normal) structure. The tail-gut and the cloaca are much smaller than normal, but not quite so small as later on. In the *Sd* / + embryo, the notochord differs from normal by a smaller calibre and irregular outline in cross-section, but it is a much more distinct organ than that in the *Sd*/*Sd* tail. The tail-gut is, on an average, smaller in cross-section than in + / + (with some overlap), but much larger than in *Sd*/*Sd*. There is no cell pyknosis beyond the normal.

At a still earlier stage (Text-fig. 3), the tail-bud is very small and there is a widely open posterior neuropore. At this stage one can hardly speak of a tail-gut, the cloaca bulging but little beyond the cloacal membrane. While the cloaca of *Sd*/*Sd* embryos is already somewhat smaller than that of normals, the difference is not sufficiently marked to be useful as a diagnostic feature. There is no appreciable size difference between the cloacae of normal and *Sd*/ + embryos. For practical purposes, the separation of + / +, *Sd*/ +, and *Sd*/*Sd* embryos at this stage is dependent on the notochord. These differences are most marked in the distal growth zone of the notochord (sections 30–50 approximately) where in the normal embryo the terminal swelling of the notochord is much larger and much more clearly defined than in *Sd*/ + and *Sd*/*Sd* embryos. The distinction between the latter two genotypes is a quantitative one, the *Sd*/*Sd* notochord, in its terminal region, being more extremely abnormal than that of *Sd*/ + embryos. However, the difference is sufficiently marked to enable the classification to be made with some confidence.

In the earliest stage examined (9 days, Text-fig. 4), the embryo is U-shaped with the posterior end of the body next to the head; there is no trace of a tail-bud yet; in most animals of this stage, the ear invaginations are not yet quite closed. At this early stage, it is difficult to be certain whether a slight reduction in size of the cloaca of putative *Sd*/*Sd* embryos can be regarded as significant. Distinction between the three genotypes can be made on the basis of the structure of the notochord, as will be discussed in more detail below. The differences between putative *Sd*/ + and *Sd*/*Sd* embryos are rather less marked than in the stage shown in Text-fig. 3, but it is believed that these two genotypes can be separated at this stage fairly accurately.

THE NOTOCHORD

In discussing the fate of the notochord in *Sd*/ + and *Sd*/*Sd* embryos, one has to keep separate those abnormalities which are present in the notochord from the start and which, at any one stage in its development, are thus visible particularly in the neighbourhood of its growth zone, the primitive streak, and certain regressive changes which supervene later on.

The general course of events has already been briefly discussed on the basis of Text-figs. 1–4; however, in these drawings, no attempt has been made to indi-

cate the presence of notochordal material except where it is clearly distinct from neighbouring structures. More details are shown in Plates 1–3. Reading from top to bottom, these are microphotographs of the 30th, 40th, 50th, &c., section as counted from the tail tip or the posterior end of the body. Reading from left to right they are + / +, *Sd* / +, and *Sd*/*Sd* litter mates respectively. The embryos of Plate 1 are of a C.R.L. of about 4 mm., those of Plate 2 of about 3 mm., and those of Plate 3 of about 2 mm. (litters 16, 22, and 25 respectively).

Starting with the + / + embryo of Plate 1, the first photograph (section 30) passes through the widened posterior end of the notochord where it is, on the left side, not yet properly separated from the paraxial mesoderm. In the following sections the calibre is gradually reduced and the outline of the sections is always approximately circular. In the *Sd* / + embryo (middle column), the growth zone of the notochord is much reduced and poor in cells; the notochord thus produced is of a much smaller calibre than normal; its outline is less regular and its structure in general less solid. Whereas in normals the notochord is usually quite separate from the paraxial mesoderm on either side in section 30 or 40, in *Sd* / + embryos this separation is not rarely delayed until section 50 or later (Table 3 below). In *Sd*/*Sd* embryos of this stage, the notochordal anomalies are much more extreme. A proper growth zone of the notochord can usually hardly be identified at all, and over much of the tail the notochord may be discontinuous from the start; it is represented by groups of scattered cells which have a tendency to attach themselves to the greatly reduced tail-gut. In sections 60–80, third column, Plate 1, the structure ventral to the neural tube represents such compound material of notochord and tail-gut.

At a somewhat earlier stage (Plate 2), the differences between the three genotypes are similar in principle, but they are rather less extreme. The growth zone of the notochord in Sd / + and particularly in *Sd*/*Sd* embryos (first horizontal row) is much reduced. However, as one follows the notochord of the *Sd* / + embryo in a cranial direction, although there is a clear size difference at first, this soon becomes rather less marked and has virtually disappeared at the bottom of the column. However, even where the calibre of the notochord is near normal, irregularities of outline and texture betray its abnormality. In the *Sd*/*Sd* embryo, there is at this level of the tail-bud a continuous notochord though it is small, is often irregular in outline and is commonly continuous with the paraxial mesoderm for some distance (see, for instance, Plate 2, figs. 31 and 32).

At a still earlier stage (Plate 3), the differences are again of a similar kind, but rather less marked. They are, however, quite consistent and the abnormals can be distinguished from their normal sibs with confidence by the abnormalities of the caudal end of the notochord; indeed, these are the only known differences between normals and abnormals at this early stage. *Sd* / + and *Sd*/*Sd* differ from each other in the degree of notochordal abnormalities. The separation of these two classes rests on the comparison with later stages and on the comparison with litters 10–12 in which all abnormals are known *Sd* / +

individuals. At this earliest stage examined, in the present investigation, the diameter of the notochord is not yet strikingly smaller than in normal embryos though measurements would probably establish a small difference. The main difference lies in the fact that the notochord remains in contact with the paraxial mesoderm for a longer distance than normal (Plate 3, figs. 40, 41, 46, and 47). As the connexions between notochord and paraxial mesoderm are dissolved again in a cranio-caudal direction as growth goes on posteriorly, the increased zone in which connexions are present in *Sd* / + and *Sd* / *Sd* embryos corresponds to a delayed separation of notochord from paraxial mesoderm in time.

TABLE 3

The first section (in multiples of 10) as counted from the tail tip or the posterior end of the trunk in which the notochord is clearly separated from the paraxial mesoderm on either side

The upper half of the table refers to litters with a mean C.R.L. of 3·0 mm. or over, the lower half to litters with a mean C.R.L. of less than 3·0 mm. Five embryos which were either damaged or sectioned in the wrong plane have not been included in this table

		Notochord distinct in section						
Litters	*Genotype*	30	40	50	60	70	80	*Mean*
2–5	+/+	3	5	..	..	..	..	36·3
14–21	+/+	3	11	..	..	..	..	37·9
2–5	*Sd*/+	..	6	6	..	..	1	47·7
14–21	*Sd*/+	1	15	10	3	..	1	46·3
6–12	+/+	5	11	3	..	..	..	38·9
22–26	+/+	2	4	..	..	..	..	36·7
6–12	*Sd*/+	..	1	8	10	1	1	56·7
22–26	*Sd*/+	..	2	4	3	1	..	53·0
22–26	*Sd*/*Sd*	..	1*	..	..	4	2	68·6

* In this embryo the notochord is separate in section 40, but connexions with the paraxial mesoderm occur again more proximally.

A rough measure of the separation process of notochord from paraxial mesoderm may be obtained by determining the distance from the tail tip at which this process is complete. In Table 3 this has been carried out to the nearest multiple of 10 sections; the process of separation is a gradual one and an uncertainty of one or two sections either way would in any case be inevitable; for the mere estimate of a mean value the present procedure is accurate enough. The material has been divided arbitrarily in groups of litters with a mean C.R.L. above and below 3·0 mm. respectively. In normal embryos of both groups the notochord is fully separated in the neighbourhood of section 38. In *Sd* / + embryos of 3·0 mm. or over, this does not happen until section 47 on an average, and in the smaller embryos not until section 54 or so. The process of separation is thus somewhat more delayed in the earlier than in the later *Sd* / + embryos. Nonetheless, a comparison of Plates 1, 2, and 3 shows that, seen as

a whole, the abnormalities of the notochord become increasingly more severe in a cranio-caudal direction. The same applies to *Sd*/*Sd* embryos. In the younger stages separation does not happen until section 70 or 80 as a rule, but the notochord produced is, at this level, a continuous structure. Later on, that is to say, more posteriorly, it becomes so abnormal structurally that its 'separation' from the paraxial mesoderm ceases to have a meaning.

The regressive changes in the notochord have been discovered by Gluecksohn-Schoenheimer (1945). As described above, the notochord of both *Sd*/+ and *Sd*/*Sd* embryos is at first a continuous structure; later it breaks down more or less completely. It is difficult to be certain to what extent this is due to cell death (degeneration) and to what extent to a mere dissociation of cells. The process will here be referred to as a 'disintegration', this being a term without a specific meaning in pathology.

The disintegration of the notochord happens similarly in *Sd*/+ and in *Sd*/*Sd* embryos, but starts earlier and is more complete in the latter. The disintegration generally begins in the cervical region whence it spreads both anteriorly in the direction of the pituitary and posteriorly to the thoracic, lumbar, and sacral segments and beyond. In the cervical region the notochord becomes discontinuous and ultimately disappears more or less completely. The details are often difficult to follow, as the notochord at this stage is flattened closely against the ventral aspect of the neural tube and still lacks the sheath which makes it so conspicuous later on. Moreover, as there are numerous cells of sclerotomic and other origin in its immediate vicinity, it is often difficult to be certain whether a given small group of cells, or a single cell, is derived from the notochord or not. Similarly, the exact limits of disintegration are sometimes difficult to determine at this stage. For that reason, no attempt will be made to give quantitative data here. The cervical notochord begins to disintegrate in some (but not all) *Sd*/+ embryos of the present series of a C.R.L. of 2·5–3·0 mm., and soon afterwards, the process spreads in a caudal direction. In *Sd*/*Sd* embryos of 3·0 mm., disintegration of the notochord is usually already more or less complete. The ultimate result is very easily seen in 12½-day embryos, as the remnants of the notochord are now lying conspicuously in the centre of the vertebrae and the intervertebral disks and are often surrounded by a rudimentary notochordal sheath. In *Sd*/+ embryos there was scarcely any trace of a notochord in the cervical region; posteriorly, there is an increasing amount of notochordal tissue, at first in small cell-nests; in the lumbo-sacral region of the embryos examined, a rudimentary notochord can often be followed continuously through 100 μ and more. However, what there is of a notochord is always grossly abnormal; there are often abrupt changes in calibre which is nearly everywhere much smaller than normal. In the 12½-day *Sd*/*Sd* embryo examined disintegration of the notochord was virtually complete except for a few cell-nests in the sacral region; this is in agreement with the findings in earlier stages.

The situation may be summed up as follows. The formation of the notochord

from the primitive streak is disturbed. A structurally abnormal notochord is formed, and these abnormalities become increasingly severe as growth proceeds in a caudal direction. While the notochord is still growing posteriorly, it starts to disintegrate in the cervical region and this process of disintegration follows in the wake of the growing primitive streak until it finally catches up with it. Both the abnormalities of formation of the notochord and its subsequent disintegration are similar in *Sd*/+ and *Sd*/*Sd* embryos, but more severe in the latter.

TAIL-GUT AND CLOACA

As shown in Text-figs. 1–4 the tail-gut of *Sd*/*Sd* and, to a lesser extent, of *Sd*/+ embryos is of a smaller diameter (but not shorter) than that of their normal +/+ sibs. Measurements for litters 14–26 are given *in extenso* in Table 4. In the stages considered the distal part of the tail-gut is approximately cylindrical. The figure given for each animal is the average diameter, in units of 4 μ, of the tail-gut as measured in projection drawings of sections 30, 40, and 50 as counted from the tail-tip; in a few cases, the mean diameter of two cross-sections only is given.

TABLE 4

Mean diameter (in units of 4 μ) of the distal region of tail-gut or cloaca as measured in projection drawings

Litter	+/+	*Sd*/+	*Sd*/*Sd*	Average diameter +/+	Average diameter *Sd*/+	Average diameter *Sd*/*Sd*
14	14 10	8 9 12 9 6	5 11	12	8·8	8
15	15 14 17	9 10 7 13 8	..	15·3	9·4	..
16	13	10 11	5 6 5	13	10·5	5·3
17	16	14 9 10 13	7	16	11·5	7
18	19 18	11 12 9 10	5 7 6	18·5	10·5	6
19	19 19	16 15 11	..	19	14	..
20	19 17	19 10 20 14 12 20	..	18	15·8	..
21	19	14	9 7	19	14	8
22	27 24	18	16	25·5	18	16
23	28	23 28 25	17	28	25·3	17
24	22	21 22 24	..	22	22·3	..
25	27	25 31	22 26 25	27	28	24·3
26	..	27	28 25	..	27	26·5

In the older litters (14–21 inclusive), the difference between +/+ and *Sd*/*Sd* embryos is very striking. The only exceptional embryo is an *Sd*/*Sd* in litter 14 with a diameter of 11 units; however, this individual is much retarded as compared with all its litter mates not only in size (C.R.L. 3·6 mm. as compared with 4·6–5·5 mm. in the others) but also in general development; its larger tail-gut diameter thus probably reflects an earlier stage in development in which that structure is still bigger in all three genotypes. The possibility must also be considered that this embryo is in fact an *Sd*/+ rather than an *Sd*/*Sd* in spite of its

very abnormal notochord. With this (probably spurious) exception, the size distributions of + / + and *Sd*/*Sd* tail-guts do not overlap. The mean of the *Sd*/ + distribution is roughly intermediate between those of the two homozygotes; the distribution overlaps that of the normal embryos to some extent; its overlap with the *Sd*/*Sd* distribution is probably less extensive.

TABLE 5

Mean diameters of tail-gut or cloaca. Condensation of data from Table 4

Litters	+/+ (*A*)	*Sd*/+ (*B*)	*Sd*/*Sd* (*C*)	*B*/*A*	*C*/*A*
14–17	14·1	9·9	6·5	0·70	0·46
18–21	18·6	13·8	6·8	0·74	0·37
22–23	26·3	23·5	16·5	0·84	0·63
24–26	24·5	25·0	25·2	1·02	1·03

The data of Table 4 are given in a more condensed form in Table 5. In the normal embryos the reduction in tail-gut diameter from 18·6 units in litters 18–21 to 14·1 units in litters 14–17 represents a step in the normal involution which that organ undergoes almost as soon as it is formed; the same is true for the corresponding reductions in *Sd*/ + and *Sd*/*Sd* embryos. The much greater diameter found in normal embryos of litters 22–26 (about 25 units) is due to the fact that, at this early stage, no tail-gut is present yet; the diameter is thus that of the cloaca, or indeed of the hind-gut (Text-figs. 3 and 4). The cloaca of *Sd*/ + embryos is of about normal size. The same is true initially of *Sd*/*Sd* embryos (litters 24–26). However, unlike the cloaca of normal and *Sd*/ + embryos, that of *Sd*/*Sd* individuals shrinks rapidly; in litters 22 and 23 it is barely two-thirds of the size of a normal cloaca and, in litters 14–21 (Table 5, Text-figs. 1 and 2) its diameter is less than one-half normal and it often lacks a lumen for long stretches. That this is a real diminution of calibre can be seen when corresponding regions of *Sd*/*Sd* embryos of different ages (such as sections 30, 50, 80, and 110 in Text-figs. 4, 3, 2, and 1 respectively) are compared with each other. In + / + and *Sd*/ + embryos, there is little or no shrinkage of the cloaca during this interval.

The shrinkage of the cloaca in *Sd*/*Sd* embryos reduces its diameter almost to that of the tail-gut, and sometimes even the adjacent segment of the colon is similarly reduced in calibre. Moreover, in *Sd*/*Sd* embryos of the ages examined, the entoderm of the cloaca does not come into contact with the overlying ectoderm to form a cloacal membrane (Text-figs. 1–4) as a more or less massive layer of mesenchyme separates the two epithelia from each other.

The mechanism of cloacal shrinkage in *Sd*/*Sd* embryos is not yet clear. One gets the impression that the tail-gut in these embryos grows at the expense of the cloaca; but this interpretation cannot be regarded as more than a suggestion. The non-formation of the cloacal membrane is obviously connected with the

shrinkage of the cloaca, the mesenchyme migrating into the space vacated. It must, however, remain uncertain whether in a stage earlier than those examined. there has been a direct contact between entoderm and ectoderm in the cloacal region. I am inclined to regard this as probable for two reasons. In a minority of *Sd*/*Sd* animals, a more or less rudimentary bladder and urethra are formed; this could hardly happen unless there was some direct (and persistent) contact between entoderm and ectoderm. Secondly, the ventral ectodermal ridge of the tail (V.E.R.) originates at the point of contact of the two germ layers; as both *Sd*/+ and *Sd*/*Sd* embryos have a fairly large V.E.R. (see below), its formation has probably been initiated by a prior contact between ectoderm and entoderm which is subsequently undone again in the majority of embryos.

THE VENTRAL ECTODERMAL RIDGE OF THE TAIL (V.E.R.)

The V.E.R. is present both in *Sd*/+ and in *Sd*/*Sd* embryos (Text-figs. 1 and 2 and Table 6). The average number of sections in which the structure appears in normal embryos agrees well with previous findings (Grüneberg, 1957*a*), being 41 sections in either case. The number of sections with a V.E.R. in *Sd*/+ embryos is significantly smaller (33·3) and that of *Sd*/*Sd* embryos a little smaller still (30·4 sections); the difference between the last two values is not statistically significant.

TABLE 6

Number of sections of 7·5 μ thickness in which the V.E.R. can be identified

The means are based on the original (ungrouped) data. The table includes litters 2–4 and 14–21, except 2 +/+ embryos which were damaged, 2 +/+ in which the orientation was faulty, and 1 *Sd*/+ in which the V.E.R. was still proximally in contact with the cloacal membrane

Sections	*16–20*	*21–25*	*26–30*	*31–35*	*36–40*	*41–45*	*46–50*	*51–56*	*Mean*
+/+	..	..	1	2	5	8	2	1	40·8
Sd/+	..	2	13	13	7	5	2	..	33·3
Sd/*Sd*	2	..	2	4	3	..	..	..	30·4

While the V.E.R. of *Sd*/+ embryos is about 20 per cent. shorter in an antero-posterior direction than that of +/+ embryos (with that of *Sd*/*Sd* embryos possibly a little shorter still), the distributions overlap broadly, and many *Sd*/+ and *Sd*/*Sd* embryos have a V.E.R. of quite normal dimensions. Moreover, it seems that the V.E.R. of *Sd*/+ embryos is often rather thicker in a dorso-ventral direction than in normal embryos. In such cases the reduction in one dimension seems to be largely compensated by an increase in another. The effect of the *Sd* gene on the V.E.R. thus seems to be comparatively trivial.

OTHER TAIL STRUCTURES

Neural tube. In the present material no abnormalities of any kind (other than regressive changes to be mentioned below) have been discovered anywhere in the neural tube. From Text-figs. 1 and 2 it might appear that in *Sd*/+ and *Sd*/*Sd* embryos the neural tube tends to reach deeper into the tail (being present in section 20 in all four abnormal specimens) than in normals, both of which do not show the neural tube until section 30. However, this is in no way borne out by the bulk of the material and apparently is a mere coincidence.

Paraxial mesoderm and somites. Mesoderm bridges between right and left are common in the tails of *Sd*/+ and *Sd*/*Sd* embryos; they are found in animals whose tail-gut and notochord are much reduced so that a gap between neural tube and tail-gut 'invites' the ingrowth of mesenchyme. Such cross-connexions involve the proximal region of the unsegmented paraxial mesoderm and/or one or two adjacent somites. Mesoderm bridges appear comparatively late in development. With one possible exception, they are confined to litters 2 and 3 and 14–18, i.e. to embryos of 10 days or over. In litters 14–18 (Table 7), mesoderm bridges are present in 11 out of 20 *Sd*/+ embryos of C.R.L. 4·0 mm. or over; and in 7 out of 9 *Sd*/*Sd* embryos of C.R.L. 3·6 mm. or over. The reduction of notochord and tail-gut happens earlier and is more extreme in homozygotes which thus start to develop mesoderm bridges somewhat before the heterozygotes. Cross-connexions do not occur at all in normal embryos. They are well known to experimental embryologists in regions where the notochord has been removed experimentally in Amphibia, &c.

TABLE 7

Crown-rump length of embryos with (+) or without (−) mesoderm bridges from right to left. Litters 14–18

Genotype		*Crown-rump length (mm.)*									
Sd/+	+	5·5	5·0	4·8	4·7	4·7	4·7	4·5	4·4	4·3	4·0	4·0
Sd/+	−	4·7	4·6	4·2	4·2	4·0	4·0	3·8	3·7	3·6		
Sd/*Sd*	+	4·7	3·9	3·8	3·8	3·7	3·7	3·6				
Sd/*Sd*	−	4·1	3·5									

REGRESSIVE CHANGES IN THE TAIL

The regressive changes which lead to the loss of the tail or part of the tail in *Sd*/*Sd* and *Sd*/+ embryos include cell pyknosis and haemorrhagic lesions (Gluecksohn-Schoenheimer, 1945), with cell pyknosis not rarely present by itself. In the present series, haemorrhagic lesions did not occur except in the $12\frac{1}{2}$-day embryos. The distribution of pyknotic foci is similar in both genotypes, but the degenerations start earlier and are more extensive in *Sd*/*Sd* homozygotes. The most typical early localization of cell pyknosis is in the centre of lumbo-sacral

somites. Another early location is in the dorsal aspect of the neural tube of the tail; this is rarely extensive and usually somewhat distal to the foci in somite centres. In the early stages of cell degeneration, the unsegmented paraxial mesoderm of the tail tip is virtually immune, and even in later stages, the tail tip often escapes destruction; later in life most *Sd*/+ animals thus have pointed tail tips whereas *T*/+ or Brachyury heterozygotes always have blunt tails as the distal segment is lost as the result of a constriction.

The ultimate destruction of axial elements in *Sd*/*Sd* homozygotes generally involves the vertebrae from the lower lumbar region onwards. In *Sd*/+ heterozygotes the lumbo-sacral vertebrae are usually quite normal though occasional mild anomalies have been noticed in previous work. The slight pyknosis which has been found in the centres of lumbo-sacral somites of some (but by no means all) *Sd*/+ embryos of 10–10½ days thus presumably does little lasting damage. In *Sd*/+ embryos of less than 10 days no cell pyknosis (other than physiological pyknosis) has been encountered. It is thus perfectly clear that in *Sd*/+ embryos, regressive changes start much later than the structural anomalies of the notochord and the reduction of the tail-gut; most of the pyknosis in the tail region develops in the 11–12½-day interval.

The situation is similar in *Sd*/*Sd* embryos. Here the first traces of cell pyknosis were encountered in the embryos of litters 21–23 (9½ days old); two of these showed slight cell degeneration in the dorsal aspect of the neural tube of the tail-bud, and the same was perhaps the case in a third specimen; a fourth had a few pyknotic granules in some lumbar somites. In all these cases pyknosis was so slight that it was discovered only after a prolonged search, and its significance in some cases is rather doubtful. In still younger *Sd*/*Sd* embryos (in which notochordal anomalies are already quite marked) no pathological cell pyknosis has been discovered. On the other hand, in older *Sd*/*Sd* embryos pyknosis soon increases in intensity.

CAUSAL RELATIONSHIPS IN THE *Sd*-SYNDROME

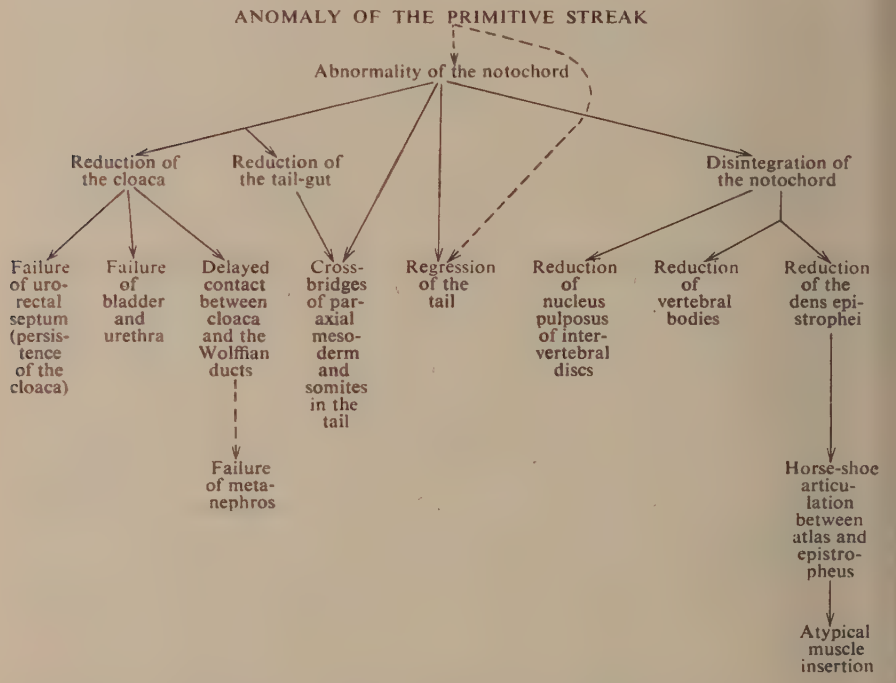
A limited attempt to unravel the causal relationships in the *Sd*-syndrome (Grüneberg, 1953) included certain features which clearly follow from the breakdown of the notochord. These are the reduction or absence of the nuclei pulposi of the intervertebral disks, the nucleus pulposus being a direct derivative of the notochord; and the reduction of the vertebral bodies for which the notochord acts as a scaffolding in the mesenchyme stage. The reduction of the vertebral bodies is most marked in the cervical region and particularly in the epistropheus where it leads to a great reduction or virtual absence of the dens. This, in its turn, involves the formation of a characteristic and fully functional 'horse-shoe' articulation between atlas and epistropheus which is quite unlike the normal pivot mechanism common to all mammals. And, yet another step removed, the longus colli muscle is shifted to an anomalous point of origin on the atlas, as its normal area of origin is now occupied by the horse-shoe articulation. Most of

these facts were independently discovered and studied by Theiler (1951–54) who arrived at virtually the same conclusions, i.e. that all these anomalies are consequences of the disintegration of the notochord. The two independent studies are thus in excellent agreement both as to the facts and as to their interpretation (though Theiler refers to the horse-shoe articulation as a 'luxation of the dens'). The present study explains why the cervical region is much more strongly affected than the rest: it is the region in which the disintegration of the notochord starts and is most complete. A somewhat similar reduction of the vertebral bodies in the absence of a notochord has been described in the chick by Watterson, Fowler, & Fowler (1954).

The data presented in this paper show beyond the shadow of a doubt that the notochord is reduced in size and abnormal in structure from the start as it emerges from the primitive streak. The severity of this process increases in a cranio-caudal direction. It has been traced back to the 2-mm. (9-day) stage of development when it is the only abnormality in *Sd*/+ and *Sd*/*Sd* embryos which has so far been detected. The subsequent disintegration of the notochord is thus not surprising and may safely be attributed to the faults in its construction. Indeed, as the notochord breaks down along its whole length, it may be suggested that it is abnormal from the very beginning; a careful study might thus be expected to reveal visible abnormalities of the notochord well before the stage to which the present investigation has been carried.

The question arises of whether the abnormality of *Sd*/+ and *Sd*/*Sd* embryos is one of the notochord as such, or whether the notochordal abnormality is in fact an expression of an abnormality of the primitive streak out of which the notochord is formed. There is, at present, no direct evidence available on the basis of which a decision can be made. However, there are one or two facts which suggest that the primitive streak is the structure primarily involved. In the first instance, the delayed separation of the notochord from the paraxial mesoderm near its posterior end gives the impression that the anomaly is not confined to the notochord, but that it also reflects some abnormality of the paraxial mesoderm cells. Perhaps the abnormality of both notochord and paraxial mesoderm cells is one of some surface property which both delays separation and, at a later stage, favours the dissociation of notochord cells and thus helps to promote its disintegration. A second point suggesting the primitive streak as the ultimate source of trouble is the fact that the *Sd*/*Sd* embryos in Gluecksohn-Schoenheimer's (1945) series—which was in several ways more severely affected than our own—had a spina bifida. Such a failure of the posterior neuropore to close might easily result from a disturbance of the primitive streak, but would be less easily explicable if the notochord were the ultimate structure at fault. In the 'pedigree of causes' given in Text-fig. 5 an anomaly of the primitive streak has thus tentatively been assumed to be at the root of the *Sd*-syndrome. The unity of the causation of the syndrome would, however, not be affected if the primitive streak should ultimately be exonerated from blame.

The regressive processes in the tail consist basically of cell pyknosis which may in the terminal stages be accompanied by a vascular breakdown. As summarized by Glücksmann (1951) the breakdown of cell groups is a widespread phenomenon in normal embryonic development, and the study of normal mouse embryos reveals its presence in numerous parts of the body at one time or another. This physiological cell pyknosis is histologically indistinguishable from that which occurs in the lumbo-sacral and caudal regions of *Sd*/+ and *Sd*/*Sd*



TEXT-FIG. 5. 'Pedigree of causes' of the *Sd*-syndrome.

embryos. Indeed, the same general type of cell pyknosis occurs in other mutants affecting the tail, such as Brachyury (*T*/+; Chesley, 1935; Grüneberg, unpublished), taillessness (*T*/*t*; Gluecksohn-Schoenheimer, 1938), vestigial-tail (*vt*/*vt*; Grüneberg, 1957*a*), and Crooked-tail (*Cd*/+; Matter, 1957). The cell pyknosis found in all these mutants is thus apparently not in itself pathological though its localization and extent is. It is certainly something quite unspecific. In *Sd*/+ and *Sd*/*Sd* embryos, cell pyknosis appears much later than the structural abnormalities of the notochord. The pyknosis is obviously the mechanism by means of which part or the whole of the tail are ultimately destroyed, but it cannot be the cause of any other (known) part of the *Sd*-syndrome. It seems reasonable

to hold the notochord responsible for the regressive changes in the tail. In the first instance, the notochordal abnormality is the first visible defect in the *Sd*-syndrome and precedes the appearance of regressive processes in time; if the notochord were not responsible, it would be necessary to invoke, *ad hoc*, an unknown agency in its stead. Secondly, cell breakdown happens where the notochordal abnormalities are most severe, i.e. near the caudal end of the body and in the tail; in *Sd*/+ heterozygotes the abnormalities are severe enough for a complete breakdown in the distal region of the tail only; in *Sd*/*Sd* homozygotes throughout the whole length of the tail and even proximal to it. The suggestion that the notochord might exert an influence over its neighbours is certainly not new in embryology. On the other hand, it is also possible that some of the regressive processes in axial structures reflect weaknesses of cells which trace back to the anomaly of the primitive streak whose existence has been suggested on other grounds; this possibility is indicated by a broken arrow in Text-fig. 5.

If we now turn to the left side of the pedigree of causes it is obvious that the reduction of the cloaca and the reduction of the tail-gut are one and the same entity. *Sd*/+ and *Sd*/*Sd* embryos differ only in degree, the reduction of the tail-gut being less marked and that of the cloaca absent or nearly so in the heterozygotes. It is obvious that the cloaca of *Sd*/*Sd* embryos is far too small for the development of a uro-rectal septum; the persistence of the cloaca is thus a direct consequence of its reduced size, a conclusion already reached by Gluecksohn-Schoenheimer (1945). The shrinkage of the cloaca also leads to what is probably a secondary separation from the cloacal membrane; if complete this will result in absence of bladder, urethra, and genital papilla; if incomplete, in a reduction of these structures. Finally, the reduction of the cloaca leads to a delay (or arrest) in the establishment of contact with the Wolffian ducts. I am inclined to hold this delay responsible for the anomalous development of the ureter buds which results in more or less complete absence of the metanephros (Gluecksohn-Schoenheimer, 1945); the latter suggestion is indicated by a broken arrow in Text-fig. 5.

It seems, then, that the striking abnormalities of the *Sd*/*Sd* homozygote (absence of rectum and anus, persistence of cloaca with reduction or absence of bladder, urethra, and genital papilla, and absence of the metanephros) can all be reduced to a common denominator, the reduction in size of the cloaca and the tail-gut. It is here suggested that there is a strong *prima facie* case for regarding the reduction of cloaca and tail-gut as a consequence of the abnormality of the notochord. There are three reasons for this suggestion. In the first instance, notochord and gut are neighbours so that an effect of one on the other is easily understandable. Secondly, the abnormality of the notochord is present before the reduction of the cloaca sets in and indeed before any tail-gut is present at all. Thirdly, and in my opinion most compellingly, there is a consistent correlation between the degree of notochordal abnormality and the degree of reduction of the cloaca and tail-gut. In the anterior parts of the body the abnormality of the

notochord is comparatively mild and the gut is not detectably involved. In *Sd*/*Sd* embryos the abnormality of the notochord becomes severe at the level of the cloaca and even before and cloaca and tail-gut are greatly reduced. In *Sd*/+ embryos, the abnormality of the notochord is less marked so that it remains below the threshold except in the tail where a rather mild reduction of the tail-gut is demonstrable. It is, of course, realized that the facts here discussed do not amount to conclusive proof that the reduction of cloaca and tail-gut are a direct consequence of the abnormality of the notochord: conclusive proof of this proposition will have to come from experimental embryology, which is thus confronted with a new problem for investigation. However, the fact that a very complex situation has been reduced to a simple common denominator to my mind strongly suggests that the causal analysis of this syndrome cannot be very far from the truth in its essentials.

It remains to mention a few minor points. The connexions across the midline between paraxial mesoderm and/or somites in the tail region which arise late in development are obviously due to a reduction in size of both the tail-gut and the notochord so that there is a gap for contact. The same phenomenon has been observed by many experimental embryologists when in amphibian larvae the notochord has been removed experimentally. The slight effect of the *Sd* gene on the ventral ectodermal ridge of the tail is presumably secondary; it may well stem in some way from the involvement of the cloacal membrane discussed above; in the absence of more detailed information, the effect has been omitted from the diagram. Finally, there are two statistical and presumably very remote effects of the *Sd* gene on the skull (Grüneberg, 1955). One of these, a tendency (in one out of two sets of data) to reduce the incidence of double foramina mentalia, had a low level of statistical significance and may well have been due to an accident of sampling. The other effect, also present in one only of the two sets of data, was highly significant; it is a reduction of the metoptic roots of the ala orbitalis of the presphenoid. The skeletal element in question thus lies in front of the anterior end of the notochord. The distance is perhaps not too great to exclude some effect of the disintegration of the notochord; the difference between the two sets of data might reflect a difference in timing and extent of the disintegration of the intra-cranial section of the notochord. However, as pointed out previously, one can hardly expect to understand the exact channels which connect a gene with its more remote manifestations. Hence these remarks are clearly of a very tentative nature.

EARLIER EMBRYOLOGICAL STUDIES OF THE *Sd*-SYNDROME

The first embryological data on the *Sd*-syndrome have been published by Gluecksohn-Schoenheimer (1945). Her descriptions and illustrations show that her embryos were about a day ahead of our series; for instance, the development of the limb-buds of her 12-day *Sd*/*Sd* embryo (fig. 2) corresponds to $12\frac{1}{2}$–13 days

in our material; a 14-day embryo (Theiler, 1951, fig. 1; obtained from Gluecksohn-Schoenheimer) by the same criterion is clearly a 15-day stage in our series, and so on. Such differences between series of mouse embryos seem to reflect differences in time of implantation; the post-implantational development seems to happen at about the same speed in all mouse stocks. Where necessary the estimated nominal age on the scale of the present data is given in brackets; unfortunately, Gluecksohn-Schoenheimer has not recorded the C.R.L. of the embryos in her paper which would have made the comparisons much easier.

In both studies the *Sd* gene was on genetically heterogeneous backgrounds with Gluecksohn-Schoenheimer's embryos more severely affected than those in the present series. For instance, her *Sd*/*Sd* embryos, in the sacral region, showed a 'failure of the neural folds to close; a cleft is visible between the neural folds covered by a large transparent bleb of the epidermis' corresponding to spina bifida in the new-born animal. This manifestation was completely absent in the present series. Similarly, the breakdown of the notochord seems to have been much more complete in Gluecksohn-Schoenheimer's series; even in her *Sd*/+ embryos, only nests of 2–3 cells of notochordal origin were found in later stages. It is thus quite clear that in her material the notochord must have been at least as seriously affected as in the present series. Nonetheless, Gluecksohn-Schoenheimer seems to have noticed only the terminal stages of the breakdown of the notochord.

> At the age of about 10½–11 [11½–12] days differences between normals and heterozygotes become apparent externally, the heterozygotes showing slight abnormalities. . . . Histologically, abnormalities in the tail of the heterozygotes may be observed slightly before they become apparent externally. . . . The entire notochord of the embryo degenerates *subsequent to the appearance of abnormal processes in the tail.* [pp. 30 and 31, italics mine.] The homozygous *Sd Sd* embryo can be distinguished from its normal and heterozygous litter mates at the end of nine [10] days after fertilization by macroscopic examination. At that stage, the tail bud begins to elongate in both the normal and the homozygote, but in the homozygote a constriction appears at the tail base. The constricted tail is shorter and thinner than the normal tail. It is characterized by haematomata of different sizes in its distal part. . . . In sections of the tail bud at nine [10] days all structures of the tail are clearly abnormal: the notochord does not appear as a continuous rod, but only traces of it are found, *while in earlier stages it appears perfectly normal.* [p. 32; italics mine.]

Gluecksohn-Schoenheimer's reference to a tail-bud in 9-day embryos, like the facts mentioned above, is evidence that her series was about a day ahead of ours. Moreover, the fact that her *Sd*/*Sd* embryos at 10 days (our style), corresponding roughly to our Text-fig. 1 and Plate 1, had macroscopic tail abnormalities, haematomata, and large numbers of pyknotic granules in all tail structures, proves that these embryos were much more severely affected than ours. Yet the author asserts that in earlier stages the notochord of these *Sd*/*Sd* embryos appeared perfectly normal. Again, in the summary (p. 38) we read: 'In both

heterozygotes and homozygotes abnormalities of the notochord and neural tube are secondary to the cell degeneration processes in the tail.'

Gluecksohn-Schoenheimer (1945) recognized abnormalities of the cloaca:

> The cloaca is abnormally small in the *Sd* homozygotes of about ten [11] days. It does not grow normally, and its separation into urogenital sinus and rectum fails to take place. Occasionally, the vesical portion of the urogenital sinus develops to a certain extent, and thus can be explained the few cases of newborns which do contain a bladder, however small. The absence of the bladder in most cases and the failure of the rectum to develop thus go back to abnormalities of the cloaca in embryos of 11 and 12 [12 and 13] days. The absence of genital papilla and anal opening are probably direct results of the absence of bladder and urethra and of the rectum. [p. 33.]

This being the only and complete reference to abnormalities of the cloaca in the embryo it seems that here, as in the case of the notochord, the later manifestations only have been noticed. The involvement of the tail-gut seems to have been overlooked completely.

Having thus consistently missed the beginning of things, it is not surprising that Gluecksohn-Schoenheimer concluded (p. 37) that '. . . a common morphological basis for the skeletal and urogenital abnormalities can thus be excluded . . .'. However, it can hardly be maintained that in her material the fully-fledged abnormalities of the notochord and of the cloaca came into being suddenly and complete, like Athene from the head of Zeus, without having passed through the earlier phases described in this paper. Indeed, Gluecksohn-Schoenheimer suggested that:

> The primary action of a gene is probably much more pervasive than is apparent from the gene's visible effects as observed with the crude morphological methods at our disposal. It is to be expected that new and different methods of study will reveal effects of genes overlooked in the first analysis. [p. 37.]

The earlier effects of the *Sd* gene described in this paper which strongly suggest a unitary origin of the syndrome have not been discovered by 'new and different' methods, but by the methods of morphology (though these were possibly a trifle less crude). However, the present author would be the last to advocate the morphological method as the panacea for all problems of developmental genetics.

SUMMARY

1. An embryological investigation of the semi-dominant gene for Danforth's short-tail in the mouse shows that, contrary to the results of earlier investigations, the syndrome can be understood as a unitary system dependent on a single root cause. The effects of the gene are similar in heterozygotes and homozygotes, but more extreme in the latter.

2. The most fundamental anomaly probably resides in the primitive streak. This gives rise to a structurally abnormal notochord with a reduced calibre. The abnormality of the notochord increases in intensity in a cranio-caudal direction.

3. At a somewhat later stage the notochord disintegrates all along its length. The process starts in the cervical region where it is also most complete.

4. As a direct result of the disintegration of the notochord there is a reduction of the nuclei pulposi of the intervertebral disks and a reduction of the centra of the vertebrae, particularly in the cervical region; the dens epistrophei is virtually absent, and as a result of this anomaly, an atypical but fully functional horse-shoe articulation is formed between the first two cervical vertebrae with yet more remote effects on neighbouring muscles.

5. The abnormality of the notochord is responsible for regressive processes (cell pyknosis) which at a later stage lead to the loss of part or the whole of the tail.

6. The abnormality of the notochord is also responsible for a considerable reduction of the cloaca and the tail-gut. The cloaca is too small for the development of a urorectal septum and hence persists; the shrinkage of the cloaca also leads to a (? secondary) separation from the cloacal membrane; this results in absence of the bladder, urethra, and genital papilla. Finally, there is an arrest or delay in the establishment of contact between the reduced cloaca and the Wolffian ducts; it is suggested that this may interfere with the growth of the ureter buds and thus, in turn, lead to the absence of the metanephros.

7. Further minor effects of the *Sd* gene include right-to-left connexions between paraxial mesoderm or somites in the tail, a slight effect on the ventral ectodermal ridge of the tail, and a statistical effect on the metoptic roots of the ala orbitalis of the presphenoid. All these effects are clearly secondary, but the mechanism of the last two of them is not understood in detail.

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EXPLANATION OF PLATES

All microphotographs are transverse sections through the tail, the tail-bud or the posterior trunk region of mouse embryos and are centred on the notochord, with the neural tube on top and the tail-gut or cloaca on the bottom of the photograph. Bouin fixation; embedded by Peterfi's method; sections 7·5 μ thick; eosin and haematoxylin; magnification $\times 335$. In each case the 30th, 40th, 50th, &c., section as counted from the tail tip, &c., has been photographed.

Plate 1

Litter 16. Nominal age 10 days. Nos. 1–6 +/+ embryo (C.R.L. 4·2 mm.). Nos. 7–12 *Sd*/+ embryo (C.R.L. 4·0 mm.). Nos. 13–18 *Sd*/*Sd* embryo (C.R.L. 4·1 mm.).

Plate 2

Litter 22. Nominal age 9½ days. Nos. 19–23 +/+ embryo (C.R.L. 2·7 mm.). Nos. 24–28 *Sd*/+ embryo (C.R.L. 3·0 mm.). Nos. 29–33 *Sd*/*Sd* embryo (C.R.L. 3·1 mm.).

Plate 3

Litter 25. Nominal age 9 days. Nos. 34–38 +/+ embryo (C.R.L. 2·4 mm.). Nos. 39–43 *Sd*/+ embryo (C.R.L. 1·9 mm.). Nos. 44–48 *Sd*/*Sd* embryo (C.R.L. 2·0 mm.).

(Manuscript received 11 : vii : 57)

H. GRÜNEBERG

Plate 1

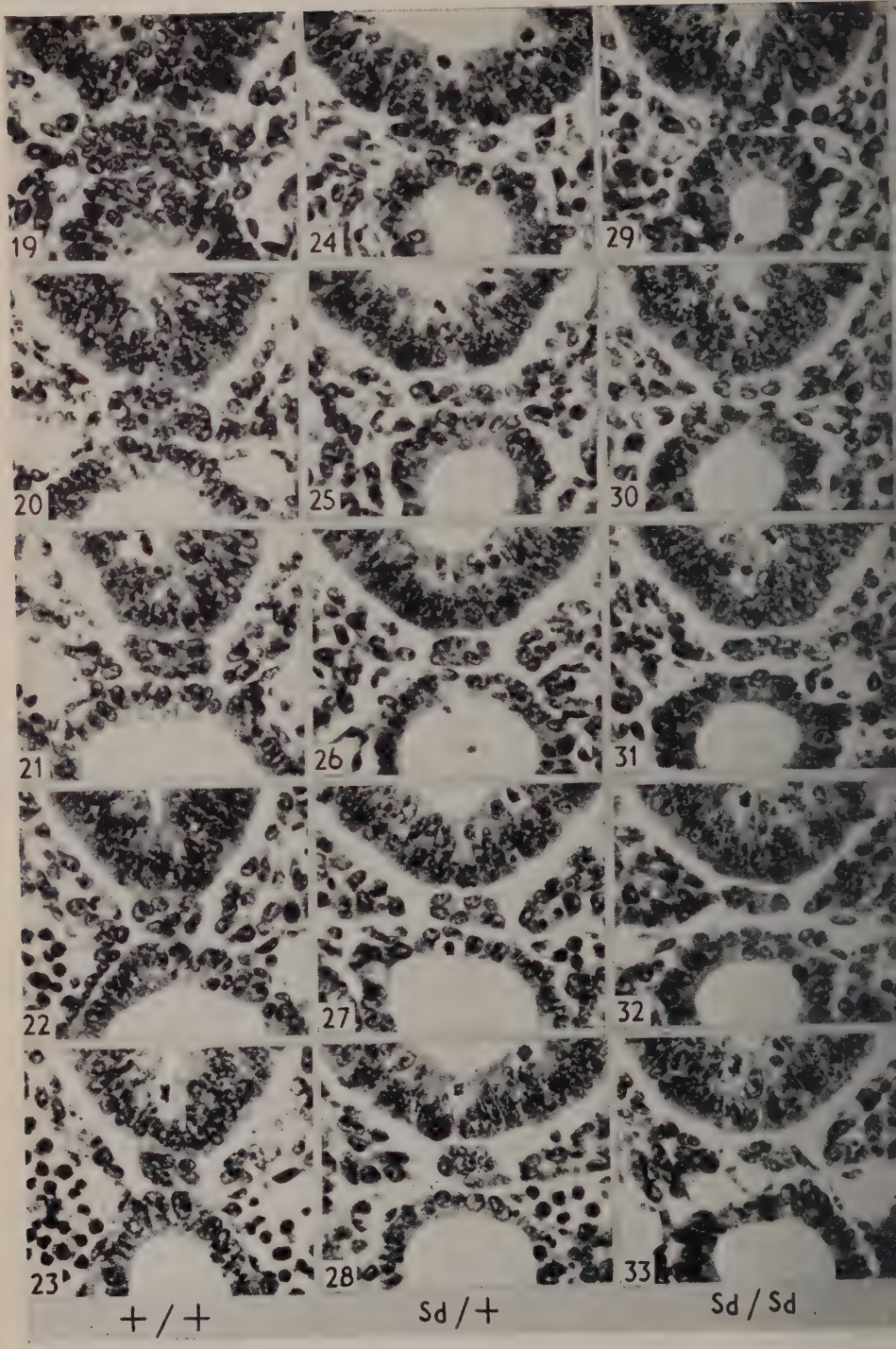

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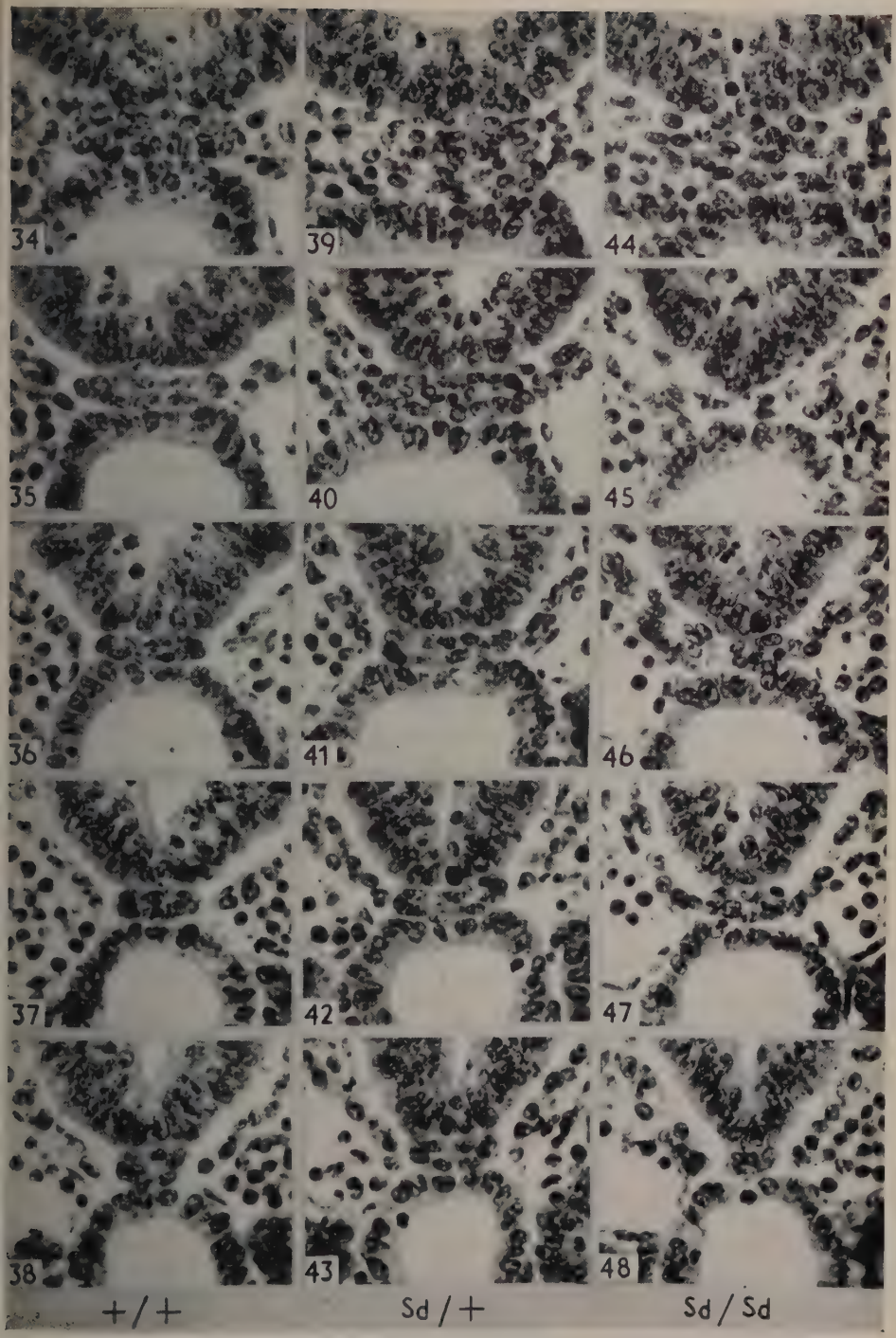
Plate 2



H. GRÜNEBERG

Plate 3

The Conversion of Yolk into Cytoplasm in the Chick Blastoderm as shown by Electron Microscopy

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WITH FIVE PLATES

INTRODUCTION

In almost all embryos yolk becomes converted into cytoplasm. It has not previously been possible to describe in any detail the morphological changes involved in this process; indeed, when the yolk drops contained within embryonic cells are examined by light microscopy they seem to remain in much the same condition until they are suddenly used up. For this reason they have frequently been considered to be nothing but 'inert, inactive' stores of food. By using an electron microscope, however, it has been possible to trace some of the morphological changes which take place in the chick when intra-cellular yolk drops are converted into cytoplasm, and to show that these are not confined to a single stage of embryonic development. Moreover, the discovery of mitochondria within the yolk drops suggests that the yolk drops are not 'inert'.

MATERIAL AND METHODS

The following stages have been examined: medium and long primitive streak (as defined by Waddington, 1932, and Abercrombie, 1950), head process, head fold, and 10–16 pairs of somites. Observations have been mainly confined to the primitive streak or to the axial tissues of the embryos, although the area opaca has also been examined. Forty-eight specimens have been used. White yolk and yellow yolk from the yolk sac of both unincubated and incubated eggs has also been studied.

Most specimens were fixed in osmic acid buffered with sodium veronal (Palade, 1952) to a pH of 7·4 and subsequently embedded in methacrylate. A few embryos were, however, fixed in potassium permanganate (Luft, 1956) at a pH of 7·4 and embedded in the epoxy resin, 'Araldite' (Glauert, Rogers, & Glauert, 1956). Ultra-thin sections were examined in a Siemens Elmiskop 1b electron microscope. Unfixed yolk and blastoderms were also studied by light microscopy.

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RESULTS

The yolk which lies beneath the blastoderm will be called *primary yolk*. The yolk drops in the blastoderm, which are usually intracellular, will be known as *secondary yolk*.

The primary yolk

Plate 1, fig. A is an electron micrograph of primary yolk from an unincubated egg. This material was examined as a smear before fixation, and was seen to consist of a liquid in which two kinds of yolk drop were floating. These three components can be recognized in the electron micrograph. The liquid seen by light microscopy corresponds with the continuous granular material (*con. ph.*). One set of yolk drops as seen by light microscopy were round, glistening, and compact; these are considered to be fatty drops (see Discussion) and are labelled *f* in the electron micrograph. The second kind were also spherical but considerably larger and translucent; within each of them were several fatty drops. I call these 'primary yolk drops' as opposed to the secondary yolk drops found within the embryonic cells (see below). In the electron micrograph it can be seen that in addition to the enclosed fatty drops the primary yolk drops contain a granular material (*gr.*) similar to the surrounding fluid (*con. ph.*) although less dense. No membranes could be seen around the primary yolk drops. These yolk drops were more common in non-incubated yellow yolk than in white yolk, or than in yellow yolk which had been incubated for 24 hours. Yolk drops of this type were never seen inside embryonic cells.

The secondary yolk

The yolk drops found within the cells of the area pellucida can be divided into three main types, the first two of which can be seen in Plate 1, fig. B, which is a section across the primitive streak of a chick blastoderm in the long primitive streak stage.

(1) Type *A* has either a dense core surrounded by a less dense region, or a granular appearance which is more or less uniform throughout. These yolk drops tend to be oval in shape after methacrylate embedding, but by shifting the block face through 90° this was found to be due to compression during sectioning. After embedding in Araldite the yolk drops were seldom compressed.

(2) Type *B* usually has a very electron dense appearance after osmic fixation and is homogeneous throughout. The irregular outline of the type *B* drops is probably an artefact (see Discussion). These drops may be derived from the fatty drops in the primary yolk.

Occasionally type *A* drops are seen to contain type *B* drops within them (Plate 5, fig. M). In most sections the type *A* drops show a wider variation in

size than the type *B* drops and are generally larger; in Plate 1, fig. B, for example, the long axis of the type *A* drops varies from $\frac{2}{3}$ μ to 3 μ, that of the type *B* drops from $\frac{1}{2}$ μ to 1 μ. The largest type *B* drops seen in the area pellucida, however, had a maximum diameter of 5 μ, but this is unusual. The average size of the yolk drops is smaller at the hind end of the primitive streak than farther forward.

(3) *Complex yolk drops.* The third type of secondary (i.e. intra-cellular) yolk drops will be called complex yolk drops, for they contain both type *A* and type *B* drops within them, as well as mitochondria, vacuoles, and various particles (Plate 2, figs. D–F). Complex yolk drops could not be recognized in the cells of living embryos examined by light microscopy, but structures closely resembling the primary yolk drops of the extra-cellular yolk could be seen.

TABLE 1

The ratio of the three main types of yolk drop at various stages

Counts were made from montages of electron micrographs. The numbers of cells involved ranged from 20 to 395. Complex drops were found in only 1 out of 13 specimens examined at the 10–16-somite stage.

Stage	*Number of yolk drops*	*Percentage of total number*		
		Complex	*Type* A	*Type* B
Primitive streak	841	5	37	60
Head process	489	4	24	72
Head fold	201	0	20	81
10–16 pairs of somites	205	1	5	92

These three types of secondary yolk drops seen in the cells of the area pellucida are similar to structures in the area opaca, although in the latter region they are larger and more plentiful. Complex yolk drops have also been seen lying between the cells of the primitive streak. Preliminary investigations show that in the area pellucida the type *B* drops are usually the most common, and they are still present when most of the complex and type *A* drops have disappeared (Table 1). In only one case were complex drops found later than the head-process stage; and in only two specimens were type *A* drops seen later than the head-fold stage. The type *B* drops also appeared to become reduced in numbers, however; for example, in the head-process stage a montage of the presumptive somite mesoderm consisting of 36 cells (the section passing through the nucleus in 11 of these) contained 103 type *B* drops. In a montage of a somite at the 12 pairs of somites stage, however, consisting of 295 cells (105 of these cut through the nucleus) only 15 type *B* drops could be found.

The conversion of secondary yolk into cytoplasm

The secondary yolk drops can be arranged in a series which suggests that they become converted into cytoplasm.

(i) *Complex yolk drops*

Within each complex yolk drop can frequently be seen a region containing granular material (*gr.*, Plate 2, fig. E) which closely resembles that in the primary yolk drops (Plate 1, fig. A). Both type *A* and type *B* yolk drops are usually present. The type *A* drops can be arranged in a series according to their structure (*A1–A5* in Plate 2, fig. E). At one end of the series is a highly granulated kind (*A1*) which resembles the granular material (*gr.*), and at the other end is a typical type *A* drop (*A5*) indistinguishable from the many which lie freely in the cytoplasm.

Other parts of the complex drops are separated from one another by internal membranes (*i.m.*, Plate 2, fig. E). Mitochondria can usually be seen inside the complex drops. Each complex drop is surrounded by a membrane. Membranous filaments are sometimes visible under the surface membrane (*m.f.*, Plate 2, fig. E).

There is evidence, set out in the Discussion, for believing that the membrane around each complex yolk drop subsequently breaks down, so that the contents of the yolk drop are released into the cytoplasm.

(ii) *Type* A *yolk drops*

After the type *A* yolk drops have formed in the complex yolk drops, see above) various changes take place. These are continued after the release of the type *A* drops into the cytoplasm. Many free type *A* drops have a dense core and a less dense outer region (Plate 1, fig. C). The series shown in Plate 3, fig. H (1–5) suggests that there is a gradual change in the structure of the core until it resembles that of the outer part of the yolk drop. The absence of a core in the 5th stage has been confirmed by examining serial sections. Eventually, the whole yolk drop when seen in transverse section usually appears to consist of a set of circular, semi-circular, or oval bodies, which will be termed *circular bodies*.

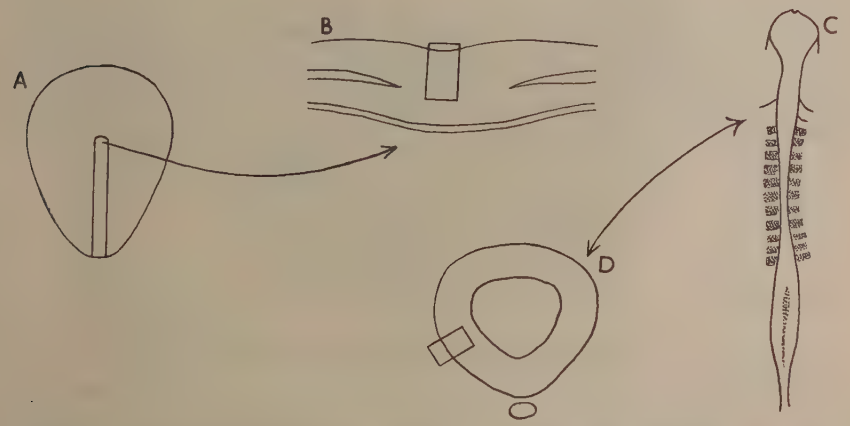
Where the circular bodies touch, they give the appearance of a latticework, but frequently they exist as discreet bodies not in contact with one another. Occasionally they appear to overlap. The circular bodies are not always the same size in adjacent yolk drops, but are usually much the same in any particular yolk drop (Plate 3, fig. I). At higher magnifications the wall of each circular body appears to be made of a membrane consisting of two dark lines separated from one another by a light line, the total thickness of the membrane being about 50 to 100 Å, (Plate 4, fig. L).

Some type *A* drops contain membranes of varying lengths (Plate 4, fig. K). Each of these membranes is about the same thickness as the wall of the circular bodies. It is possible therefore that some at least of the circular bodies are really tubules seen in transverse section. In some yolk drops both membranes and circular bodies are visible in the same section (Plate 4, fig. L).

The type *A* drops are usually surrounded by a membrane (Plate 3, fig. J and Plate 4, fig. L) which has the same structure as the walls of the circular bodies.

In some cases where the yolk drop has become completely converted into circular bodies the outer membrane appears to have broken down (Plate 3, fig. J). The contents of the yolk drop have therefore just been liberated into the cytoplasm. They closely resemble the endoplasmic reticulum.

Not all the type *A* drops become converted into circular or tubular structures, however. In some cases yolk drops are found which contain many tiny particles lying separately from one another. These will be known as *micro-particles*; they are about 100 to 150 Å in diameter. In these type *A* yolk drops type *B* yolk drops are frequently found (Plate 5, fig. M); the surrounding membranes are usually double, each part consisting of two dark lines separated by one light line.



TEXT-FIG. 1. A, diagram of area pellucida at the full length primitive streak stage. B, diagram of a transverse section across the primitive node. The rectangle shows the position in the embryo of the sections illustrated in Plate 1, fig. B and Plate 5, fig. O. C, diagram of an embryo with 11 pairs of somites. D, diagram of a section across the hind-brain of the embryo shown in C at the level indicated by the arrow. The rectangle demonstrates the position in the embryo of the section illustrated in Plate 5, fig. P.

These micro-particles in the yolk drops closely resemble similar structures in the cytoplasm, which will be called *cytoplasmic micro-particles*. After the type *A* drops have disappeared the number of these cytoplasmic micro-particles rises, in some regions at least. For instance, Plate 5, fig. O, shows part of the presumptive neural-plate ectoderm in the primitive streak stage (see Text-fig. 1 A, B). Plate 5, fig. P is a section across the hind-brain of an embryo at the stage of eleven pairs of somites (see Text-fig. 1 C, D). In the later stage the type *A* drops have completely disappeared and the number of cytoplasmic micro-particles has increased.

In Plate 2, fig. G, a mitochondrion-like body lies between the two layers of the outer membrane. Mitochondria were found within all varieties of type *A* drops.

(iii) *Type* B *yolk drops*

The type *B* drops have been examined mainly after osmic acid fixation and methacrylate embedding. Internally the type *B* yolk drops consist of a regularly arranged and apparently continuous substance. Heavily osmiophilic granules are also present, especially around the edge (Plate 5, fig. N). After fixation in permanganate, membranes were visible around the type *B* drops although this reagent failed to fix the 'continuous substance' (Plate 3, fig. J).

When they lie freely in the cytoplasm type *B* drops possess a more irregular outline than when they are forming part of a complex yolk drop. In serial sections taken through a single type *B* drop it was found that the irregularity extended over the whole surface. To make any accurate assessment therefore of the changes in size of the type *B* drops during the development of the embryo, it would be necessary to carry out a series of reconstructions at various stages. Nevertheless, I gained the impression that the type *B* drops became smaller as the embryo grew older. Evidence will be presented in a subsequent paper that they also become reduced in numbers.

DISCUSSION

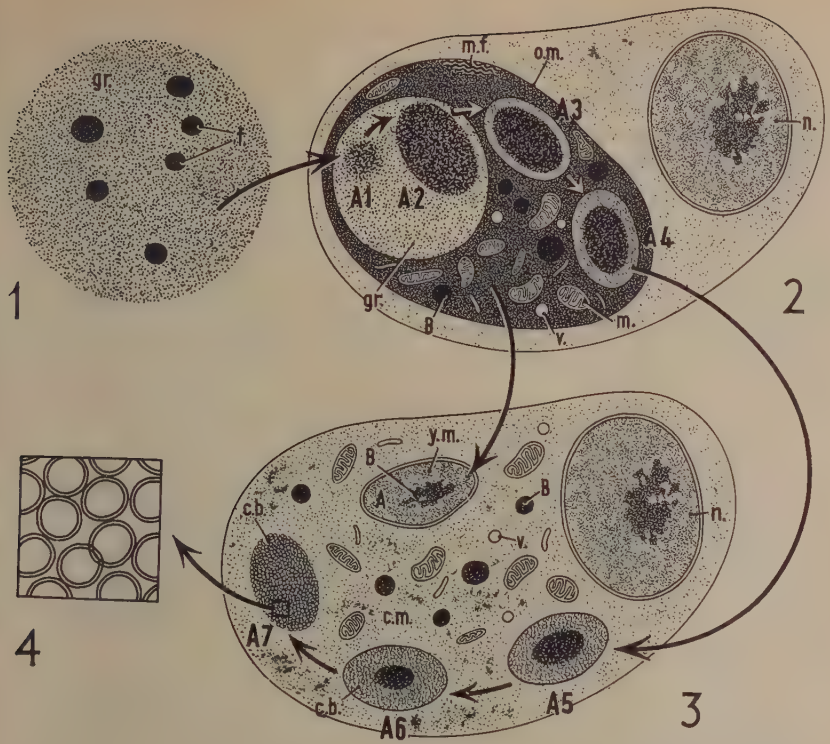
The conclusions which will be reached are summarized in Text-fig. 2.

The identity of the secondary yolk drops

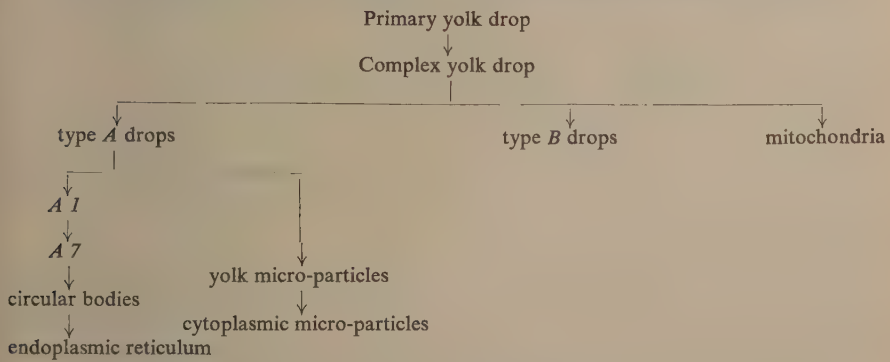
The secondary (or embryonic) yolk drops described in this paper were found in the area pellucida; they are considered to be partially converted yolk, for two reasons. First, these structures closely resemble similar ones in the area opaca which is a region containing so many yolk drops that it is tinged yellow by them when alive; the resemblance is apparent when the tissues are examined both by light and electron microscopy. Secondly, some of the secondary yolk drops contain a granular material which is similar to the granular material in the primary yolk drops (cf. *gr.* in Plate 1, fig. A and Plate 2, fig. E).

Yolk is present in the blastodisc cells from the beginning of cleavage (Konopacka, 1933). It is generally accepted that the cells of the area opaca take up the primary yolk by phagocytosis (Remotti, 1927; Schechtman, 1956), but there is little information as to whether the area pellucida cells do so too.

Complex yolk drops are believed to be derived from the primary yolk drops (see below). Complex yolk drops could not be recognized in cells studied by light microscopy, and have not been described by previous workers; they are indistinguishable from primary yolk drops under these conditions. From the present study, however, it can be seen that yolk drops with the same structure as the primary yolk drops are not present inside the embryonic cells, at least at the stages examined.



TEXT-FIG. 2. Diagram to summarize the way in which yolk appears to be converted into cytoplasm in the cells of the area pellucida. The transformations proposed are as follows:



1. *Primary yolk drop f.*, fatty drop; *gr.*, granular material.

2. *Complex yolk drop* within a cell. *o.m.*, outer membrane of yolk drop; *n.*, cell nucleus. The type *B* drops are probably derived from the fatty drops of the primary yolk drop. The type *A* drops (*A1*, *A2*, *A3*, *A4*) appear to develop from the granular material (*gr.*). Mitochondria (*m.*), membranous filaments (*m.f.*), and small vesicles (*v.*) are also present.

3. The same cell after the contents of the complex yolk drop have entered the cytoplasm. Many type *A* drops (*A5–A7*) become converted into circular bodies (*c.b.*). These may later become endoplasmic reticulum. Others (*A*) consist of yolk micro-particles (*y.m.*) which subsequently may become cytoplasmic micro-particles (*c.m.*).

4. Higher magnification of circular bodies to show the structure of their walls.

The conversion of the secondary yolk drops into cytoplasm

It has been shown that the number of yolk drops in the area pellucida gradually falls, until by the stage of 10–16 pairs of somites they have practically disappeared. This gradual reduction in the numbers of yolk drops has been reported previously by Konopacka (1933), Knorre (1951), and Lavarack (1957).

On the evidence listed below the complex drops are believed to be derived from the primary yolk drops and to give rise to some at least of the type *A* and type *B* drops. An alternative hypothesis would be that the complex drops are the result of a collecting together of the type *A* and type *B* drops, together with other cellular components such as mitochondria. There are four lines of evidence.

1. Each complex drop contains granular material similar to that in the primary yolk (cf. Plate 1, fig. A and Plate 2, fig. E).
2. Each complex drop is surrounded by a membrane; if the second hypothesis were accepted it would be necessary to postulate that a membrane arose and encircled a group of type *A* and type *B* yolk drops within the cell. If the first hypothesis is correct, however, a more likely proposition can be put forward, namely, that the membrane arose at the interface between the yolk drop and the surrounding cytoplasm.
3. Complex drops, though plentiful in the primitive-streak and head-process stages, were found only once in later stages; type *A* and type *B* drops were, however, still present. This would be expected if the complex drops were being broken down into type *A* and type *B* drops.
4. The breakdown of yolk drops has been reported by Celener (1945), and by Grodzinski (1947) who grew yolk sac endoderm cells in tissue culture. Grodzinski reported that the membrane around the yolk drop broke down and the contents were liberated into the cytoplasm.

The chemical nature of the yolk drops

According to Grodzinski (1947) the primary yolk contains fatty drops which may either float freely in a liquid protein or lie within larger yolk drops. It is concluded, therefore, that the heavily osmiophilic bodies shown in Plate 1, fig. A, are these fatty drops.

Fatty drops are also known to occur in the cytoplasm of the area pellucida (Fraser, 1956) and within some of the secondary (intra-cellular) yolk drops (Konopacka, 1933; Thomas, 1938). They have been reported to persist after the rest of the secondary yolk has disappeared (Konopacka, 1933). The type *B* drops fulfil these conditions and are therefore judged to be predominantly fatty. Moreover, they resemble similar structures seen in electron micrographs of tissues known to have a high fat content (Lever, 1957). The appearance of the fatty drops in the primary yolk is, however, not identical with that of the type *B* drops. It is concluded therefore that, although both are fatty, they do not have exactly the same structure.

Apart from the fatty drops and a small amount of carbohydrates, primary yolk consists largely of proteins or lipo-proteins (Needham, 1931, 1950; Konopacka, 1933; Thomas, 1938; Grodzinski, 1947). The type *A* drops appear to be derived from the fluid part of the primary yolk drops (*gr.*, Plate 1, fig. A) and are therefore considered to be predominantly protein or lipo-protein mixtures.

The conversion of the type A *drops into cytoplasmic structures*

The present investigation shows that the formation of the type *A* drops may take place within the complex drops. This appears to be the process described by Konopacka (1931) for the yolk drops of the area opaca cells. She found that the protein material formed into large granules, after which the whole yolk drop broke down and the granules became vacuolated and disappeared.

(i) *The circular bodies*

The transformation of the type *A* drops into the circular bodies may in some cases start inside the complex drops, but in others take place entirely in the cytoplasm after the complex drops have broken down. It is also possible that some of the type *A* drops lying freely in the cytoplasm are derived directly from the ingestion of the proteins of the primary yolk rather than from the dispersal of the contents of the complex yolk drops; in such cases, however, the membrane around the type *A* drops must have arisen in a different way.

The walls of each circular body appear to consist of a membrane composed of two dark lines separated by a light line. Membranes of this type were first described by Robertson (1957, 1958) in nerve-fibres. Because they contain membranes it is considered that the type *A* drops consist predominantly of lipo-proteins.

The circular bodies appear to be released into the cytoplasm. They closely resemble the endoplasmic reticulum; it is possible, therefore, that they contribute to this system although there is no evidence of any rise in the amount of endoplasmic reticulum just after the yolk drops have disappeared.

(ii) *The micro-particles*

Some of the type *A* drops appear to be transformed into micro-particles rather than into circular bodies. The genesis of these yolk drops has not been followed, but it is possible that they arise from those parts of the complex drops whose fate has not been traced, for instance, the dense material (*d*) seen in Plate 2, fig. D. The micro-particles in the yolk drops are of comparable size and appearance to the cytoplasmic micro-particles. After the type A drops have disappeared there is a striking increase in the numbers of cytoplasmic micro-particles, especially in the neural tube. It is suggested, therefore, that the yolk micro-particles enter the cytoplasm and become converted into cytoplasmic micro-particles.

The cytoplasmic micro-particles are believed to be ribonucleoproteins for two reasons. First, there is evidence that similar structures found in other tissues

are ribonucleoproteins (Palade, 1955; Palade & Siekevitz, 1956). Secondly, there is a rise in the amount of ribonucleoproteins in the neural tube (Gallera & Oprecht, 1948; Brachet, 1950; Lavarack, 1957) at the time when the numbers of cytoplasmic micro-particles are increasing. According to Lavarack this rise in ribonucleoproteins occurs as the yolk drops break down. It is suggested, therefore, that yolk micro-particles become converted into ribonucleoproteins at the time when they are released into the cytoplasm. It is relevant to note here that there is some biochemical evidence that the yolk of the hen's egg, as well as that of other vertebrates, may become converted into ribonucleoproteins (Brachet, 1950).

The fate of the type B *yolk drops*

According to Grodzinski (1947) lipase breaks down the surface of the fatty drops in the primary yolk and causes it to fuse into larger bodies. It seems unlikely that the type *B* drops in the area pellucida cells fuse together in this way, however, for as the embryos became older, the type *B* drops appeared to become smaller. They also appeared to become fewer in number, so that it is possible that their substance gradually seeps away into the cytoplasm. Here perhaps it remains as small isolated granules, or it is possible that it becomes attached to other structures in the cytoplasm.

Grodzinski (1947) concluded that fatty drops become transformed from glycerides to phosphatides. Fraser (1956) has suggested that cytochrome oxidase is attached to the fatty drops in the young chick blastoderm, but according to Spratt (1952) this enzyme may be associated with the mitochondria in the chick.

The origin of the mitochondria found within the yolk drops

Mitochondria have not previously been reported to be present inside yolk drops. The present work shows that they may occur within the complex drops and within the type *A* drops. There appear to be four possible explanations:

1. *The mitochondria are maternal ones.* Brambell (1925), who studied oogenesis in the fowl, concluded that much of the yolk is formed directly from mitochondria. The mitochondria in the intracellular yolk drops might therefore have been present in the yolk drops from the time of oogenesis.
2. *The mitochondria have formed inside the yolk drops.* This might take place by the conversion of the circular bodies.
3. *The mitochondria have formed by an invagination* and subsequent proliferation of the membranes surrounding the yolk drops.
4. *The mitochondria have migrated into the yolk drops* from the surrounding cytoplasm.

It may be noted here that occasionally in yolk drops which are surrounded by double membranes, small sac-like objects can be seen lying between the two membranes (Plate 2, fig. G). These may be mitochondria either forming from

the membranes as in hypothesis 3, or migrating in from the cytoplasm as in hypothesis 4. The problem will be discussed more fully in a subsequent paper.

It is possible that the mitochondria perform an important function in the digestion of yolk, for they are known to contain large numbers of enzymes (Gustafson, 1954).

SUMMARY

1. Three main types of yolk drop are present in the area pellucida of the chick blastoderm. They have been given the names 'complex yolk drop', 'type *A* yolk drop', and 'type *B* yolk drop'. Together they have been called *secondary yolk* to distinguish them from the extra-embryonic or *primary yolk*.

2. It is concluded that the complex yolk drops are early stages in the conversion of yolk. They contain granular material which resembles that found in the primary yolk. Type *A* drops are present in the complex yolk drops and appear to be formed from the granular material. Type *B* drops, mitochondria, vacuoles, and membranes are also found within the complex drops.

3. The membranes around the complex drops appear to break down, releasing the contents of the yolk drops into the cell.

4. Most type *A* drops appear to become converted into a number of circular, or possibly tubular, bodies. These are believed to be predominantly lipoproteins. It is concluded that the membrane around each type *A* drop breaks down and these circular bodies are then released into the cytoplasm where they may contribute to the endoplasmic reticulum.

5. Other type *A* drops are composed of numbers of micro-particles. They closely resemble the cytoplasmic micro-particles and may become part of them. Evidence is presented for considering these micro-particles to be precursors of ribonucleoproteins.

6. The type *B* drops persist in the cytoplasm after the complex drops and the type *A* drops have almost completely disappeared. Evidence is presented for considering them to be fatty drops.

7. Primary yolk from the hen's egg has also been examined.

ACKNOWLEDGEMENTS

It is a pleasure to thank Dr. J. David Robertson for his patience in instructing me in the various aspects of electron microscopy. I am also greatly indebted to him, to Professor J. Z. Young, F.R.S. (in whose department the work was carried out), and to Dr. A. J. Marshall for the critical interest which they have taken in this work. I am also most grateful to Miss Rose Smith and Mr. J. Pettitt for technical assistance, and to Miss E. R. Turlington for Text-fig. 2.

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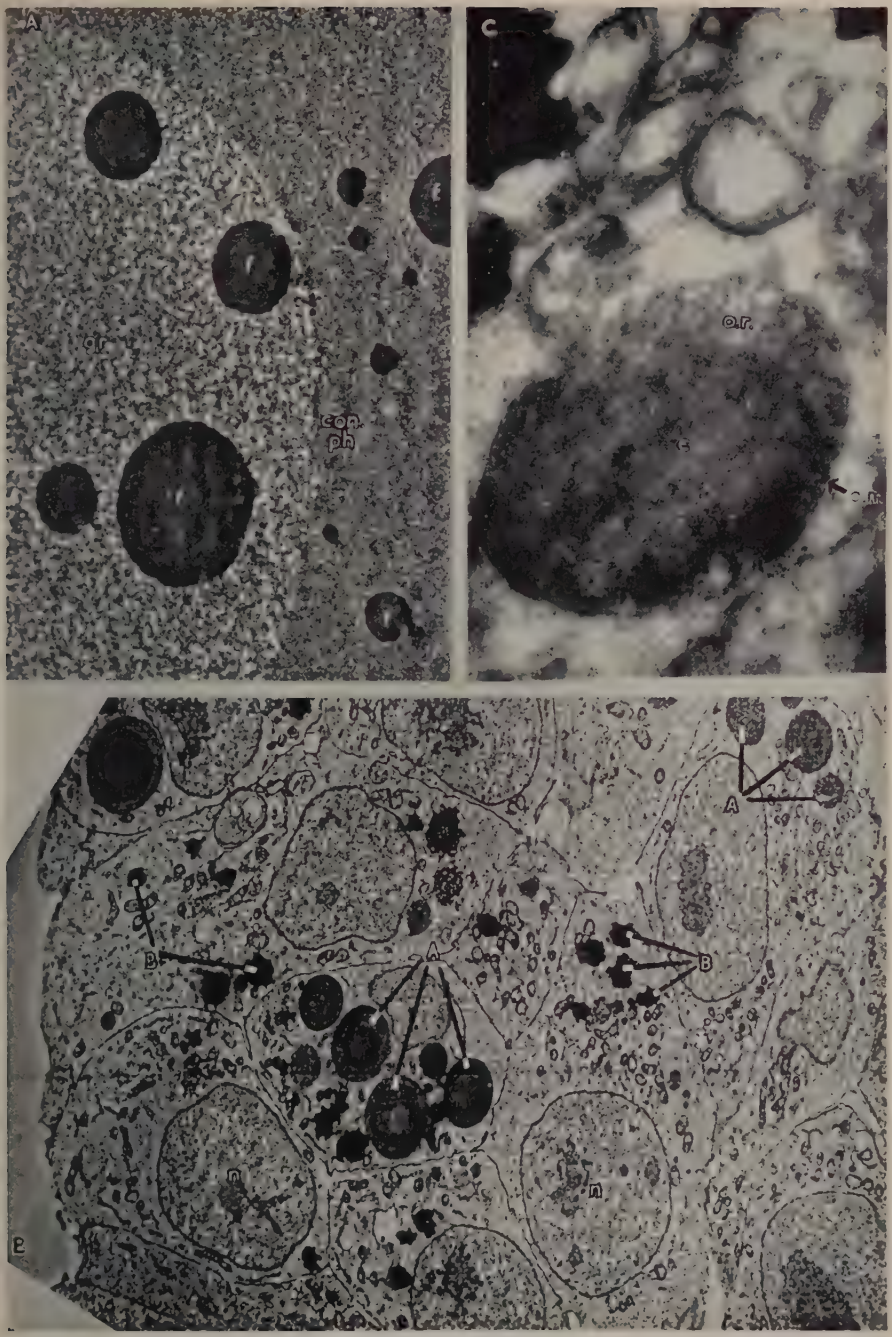
EXPLANATION OF PLATES

Plate 1

Fig. A. Yolk from an unincubated hen's egg showing on the left the edge of a primary yolk drop and on the right the continuous liquid phase of the yolk (*con. ph.*). Fatty drops (*f*) are found



R. BELLAIRS

Plate 1

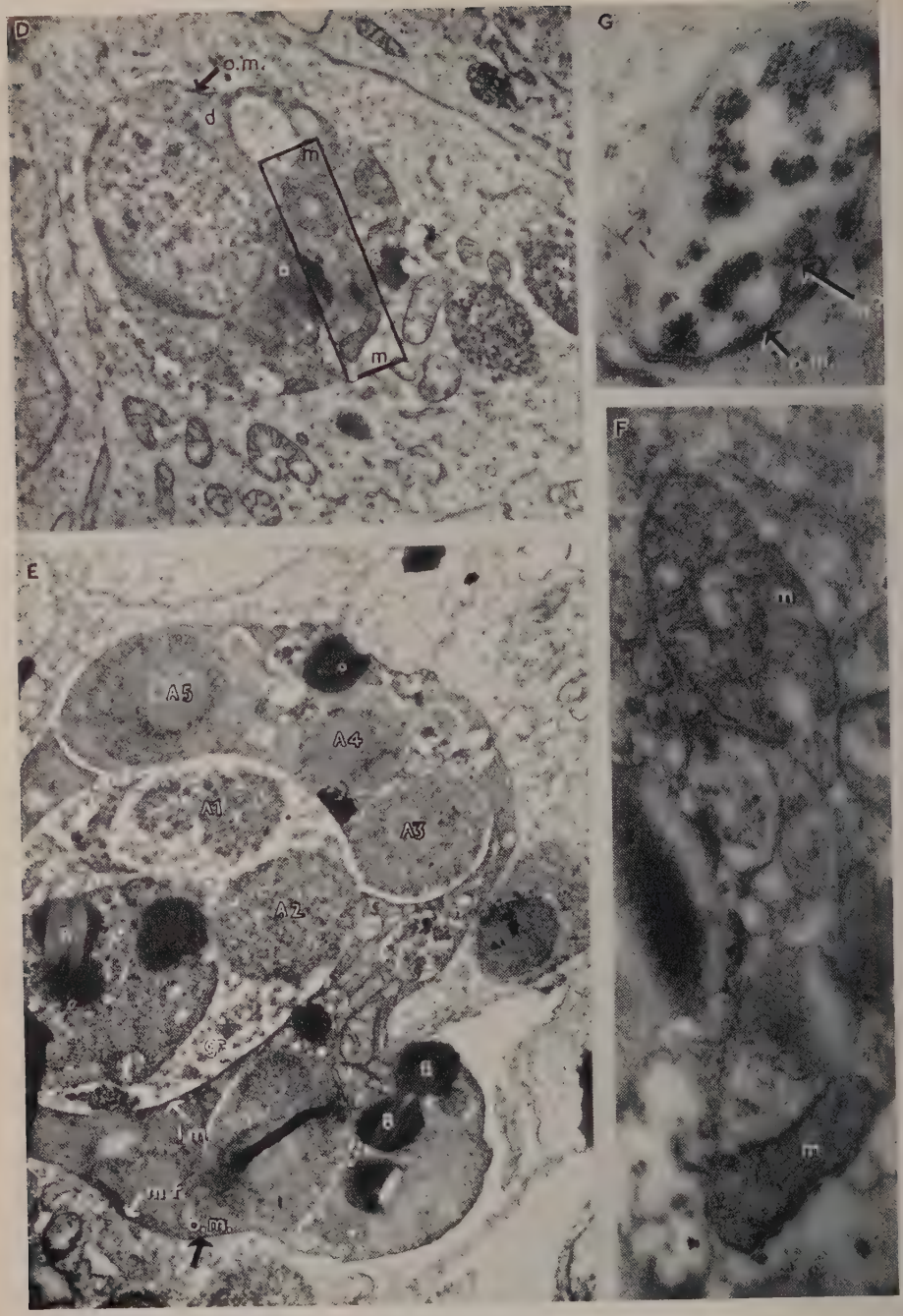


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Plate 2

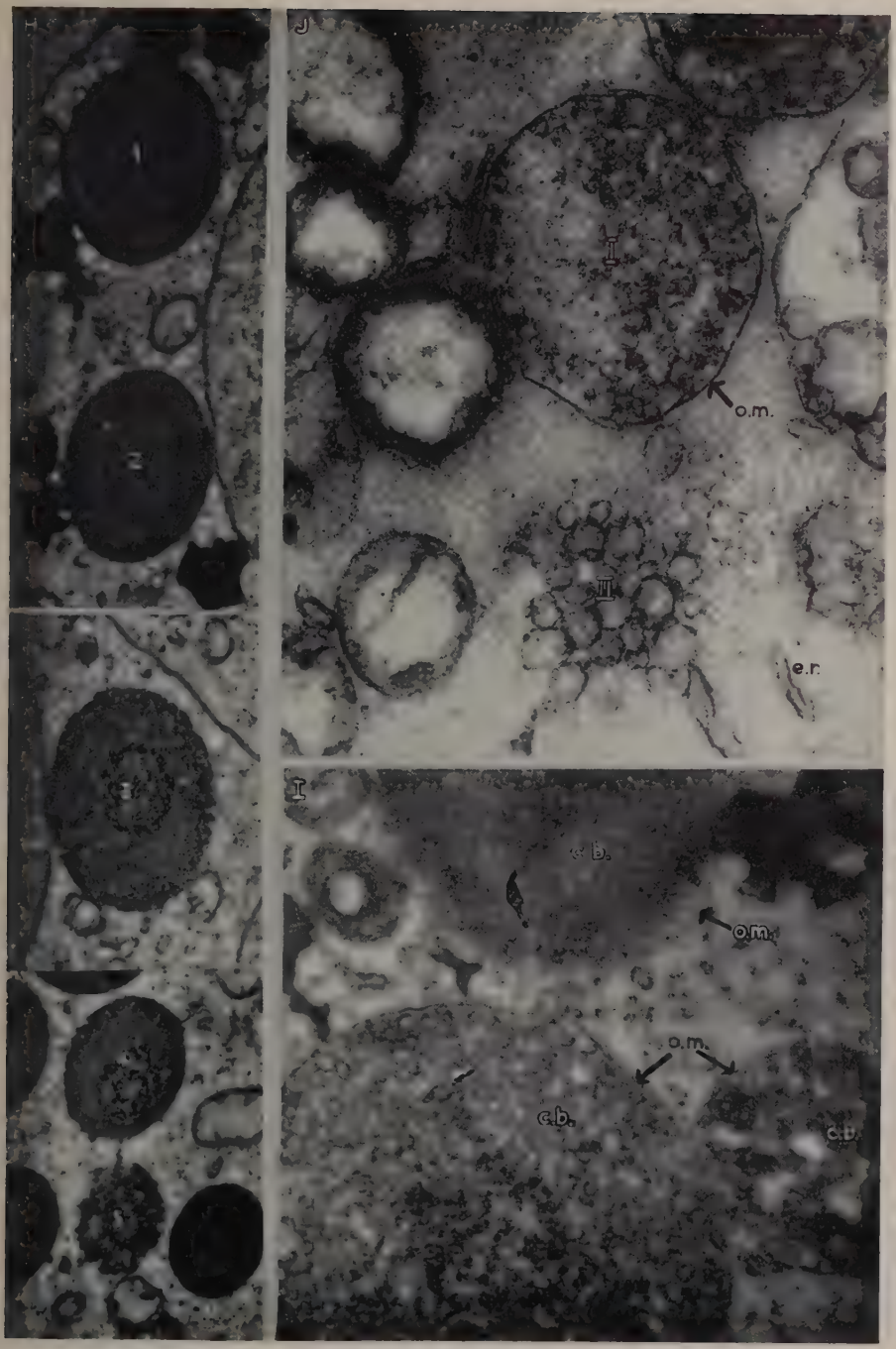

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Plate 3

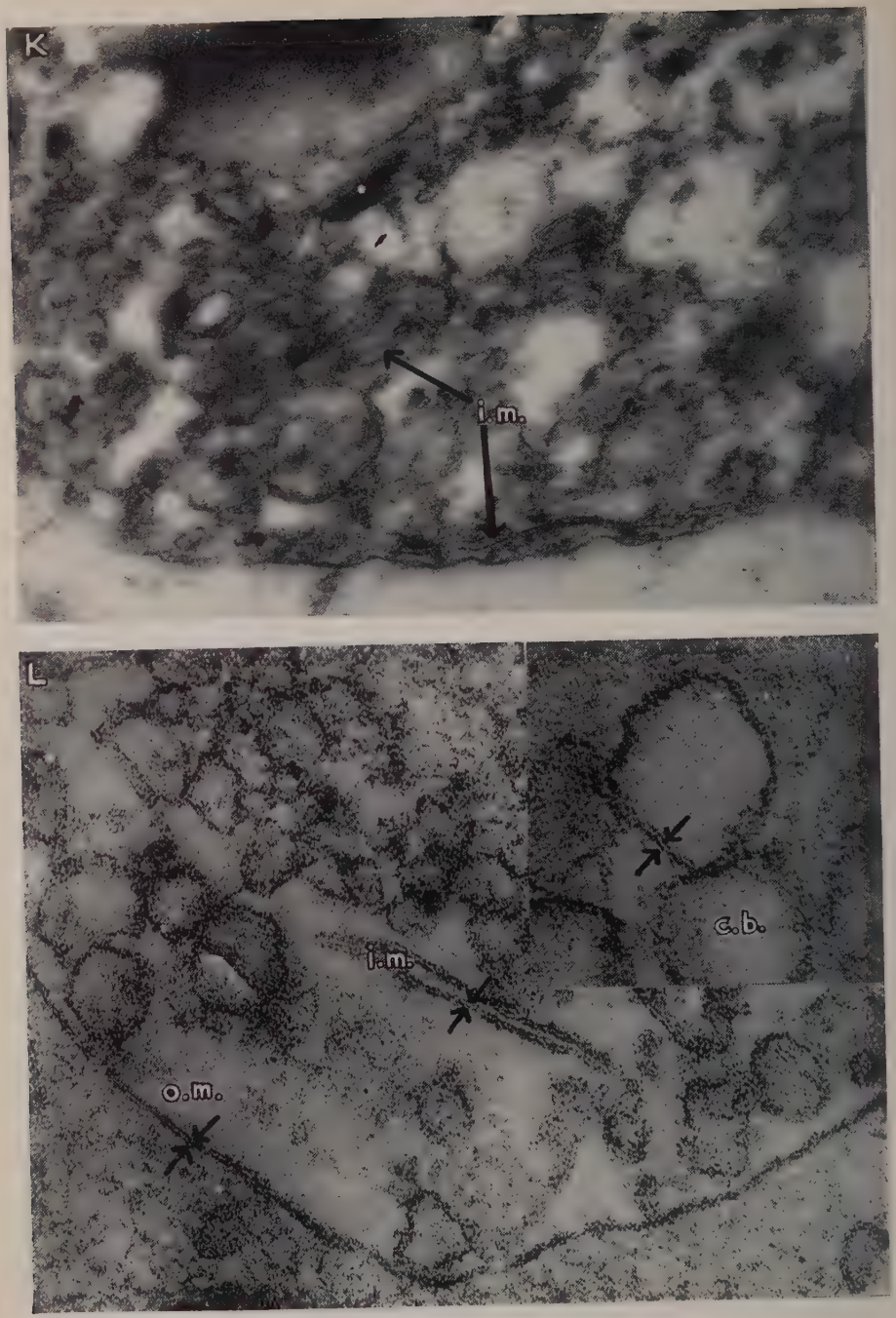

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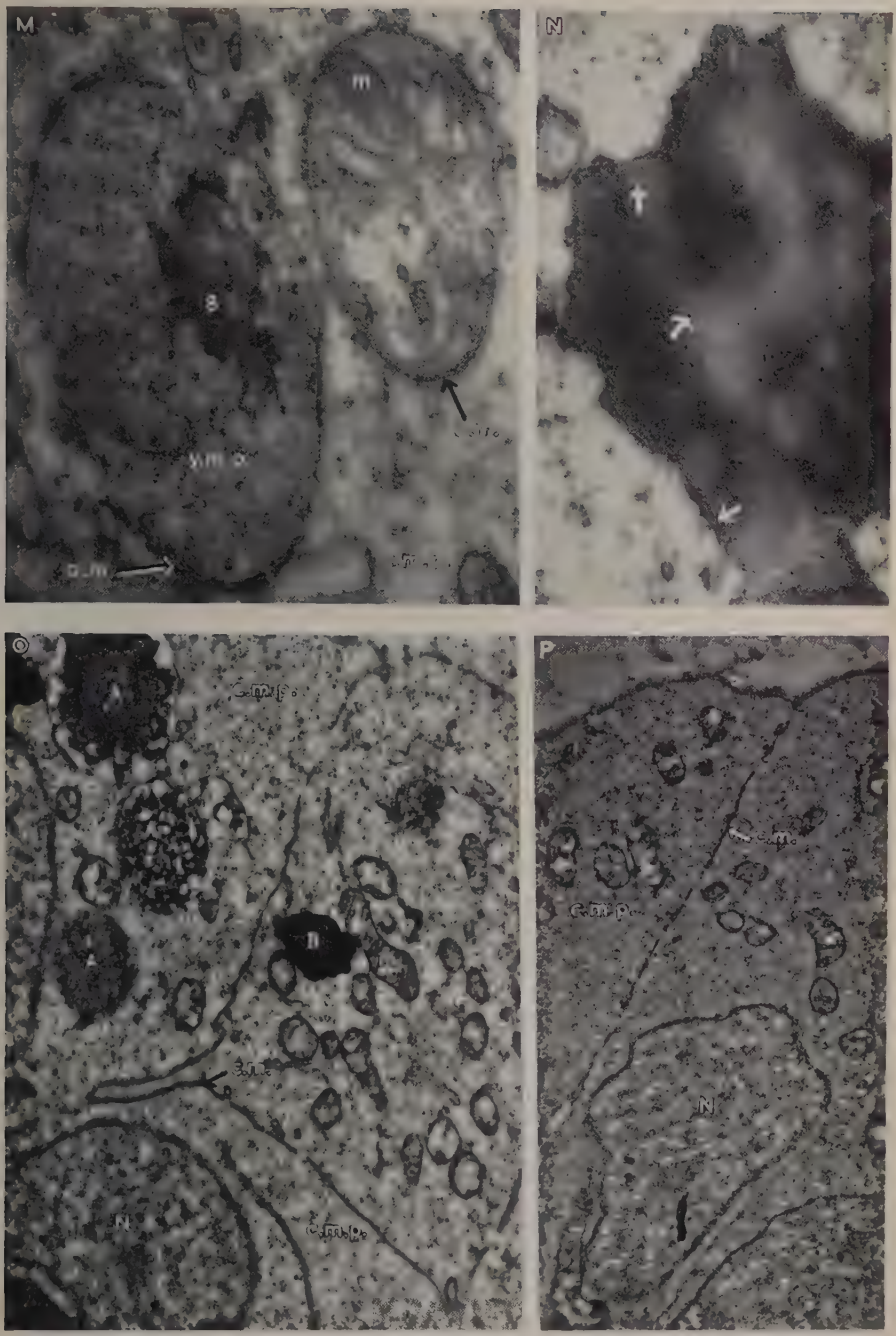
Plate 4



R. BELLAIRS

Plate 5

both within the primary yolk drops and lying freely in the liquid. The primary yolk drops also contain a granular material (*gr.*). Osmic and methacrylate. Magnification: ×11,000.

Fig. B. Transverse section through the primitive node of a long primitive streak stage blastoderm (see Text-fig. 1 A, B). The dorsal side is to the left. *A*, type *A* drop; *B*, type *B* drop; *n*, nucleus. Osmic and methacrylate. Magnification: ×3,600.

Fig. C. Type *A* yolk drop showing dense core and less dense outer region. *c*, core; *o.r.*, outer region; *o.m.*, outer membrane. Osmic and methacrylate. Magnification: ×26,000.

Plate 2

Figs. D and E. Two complex yolk drops. *o.m.*, outer membrane; *i.m.*, internal membranes; *gr.*, granular material resembling that of the primary yolk drops; *A1*, *A2*, *A3*, *A4*, and *A5*, apparent stages in the formation of type *A* drops from the granular material; *B*, type *B* drop; *m.*, mitochondrion; *m.f.*, membranous filaments; *d.*, dense material. Osmic and methacrylate. Magnification: *D*, ×11,000; *E*, ×8,000.

Fig. F. Enlargement of the rectangle outlined in fig. D to show the mitochondria. Osmic and methacrylate. Magnification: ×36,000.

Fig. G. Type *A* yolk drop. At one side a mitochondria-like body can be seen between the two layers of the surrounding membrane. Osmic and methacrylate. Magnification: ×14,000.

Plate 3

Fig. H. Series of type *A* yolk drops showing their conversion into a group of circular bodies (*A5*). Osmic and methacrylate. Magnification: ×9,000. The differences in size depend mainly on whether the section passes through the middle of the yolk drop or not.

Fig. I. Three type *A* yolk drops each with different-sized circular bodies (*c.b.*) and each surrounded by a membrane (*o.m.*). *e.r.*, endoplasmic reticulum. Osmic and methacrylate. Magnification: ×30,000.

Fig. J. Type *A* yolk drops. In *I* an outer membrane (*o.m.*) is present. In *II* this membrane appears to have been broken down. *e.r.*, endoplasmic reticulum. Permanganate and Araldite. Magnification: ×36,000. Three type *B* yolk drops are present in the upper half of the left side of the figure.

Plate 4

Fig. K. Edge of a type *A* drop containing internal membranes (*i.m.*). Osmic and methalcrylate. Magnification: ×54,000.

Fig. L. Edge of type *A* drop containing membranes (*i.m.*) and surrounded by an outer membrane. (*o.m.*). The regions between the paired arrows indicate the width of each membrane, which consists of two dark lines separated by a light line. Permanganate and Araldite. Magnification: ×130,000. Inset: circular body (*c.b.*) from same specimen. Magnification: ×176,000.

Plate 5

Fig. M. Two type *A* drops, one with an enclosed type *B* drop, the other with an enclosed mitochondrion. Both yolk drops are surrounded by two membranes and contain within themselves micro-particles which closely resemble the cytoplasmic micro-particles. *B*, type *B* yolk drop; *o.m.*, outer membranes; *m.*, mitochondrion; *y.m.p.*, yolk micro-particles; *c.m.p.*, cytoplasmic micro-particles. Osmic and methacrylate. Magnification: ×28,000.

Fig. N. Type *B* yolk drop. The heavily osmiophilic granules are indicated with arrows. Osmic and methacrylate. Magnification: ×74,000.

Fig. O. Presumptive neural-plate ectoderm of a primitive streak stage blastoderm (see Text-fig. 1A). Yolk drops are present and cytoplasmic micro-particles are sparse. *A*, type *A* yolk drop; *B*, type *B* yolk drop; *c.m.*, cell membrane; *n.*, nucleus; *m.*, mitochondrion; *c.m.p.*, cytoplasmic micro-particles. Osmic and methacrylate. Magnification: ×11,000.

Fig. P. Section through the hind-brain of an embryo at the stage of 11 pairs of somites (see Text-fig. 1 C, D). Yolk drops have disappeared and the number of cytoplasmic micro-particles has increased. *n.*, nucleus; *c.m.*, cell membrane; *c.m.p.*, cytoplasmic micro-particles. Osmic and methacrylate. Magnification: ×11,000.

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Interaction ectoderme–mésoderme dans la formation des invaginations uropygiennes des Oiseaux

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AVEC DEUX PLANCHES

INTRODUCTION

La glande uropygienne ou glande du croupion est, avec les cellules d'aspect glandulaire de l'oreille, la seule glande cutanée des Oiseaux. Sa première ébauche apparaît chez l'embryon de Canard au 11ème jour de l'incubation sous forme d'une double invagination ectodermique dorsale au niveau des vertèbres sacrées.

Nous avons montré (Gomot, 1956*a*) que la moitié dorsale du bourgeon caudal d'embryons de Canard explantée sur le milieu de culture gélosé standard de Wolff & Haffen (1952), forme des invaginations uropygiennes ou, au moins, des fentes ectodermiques lorsqu'elle provient d'embryons ayant au moins 8 jours d'incubation. Il y a donc, à partir de cet âge, autodifférenciation *in vitro* de la moitié dorsale du bourgeon caudal isolée du reste de l'embryon. De même lorsque nous enlevons (Gomot, 1956*b*) l'ectoderme et le mésoderme sous-ectodermique dorsaux du bourgeon caudal d'embryons du même âge — 8, 9, 10 et 11 jours — et que nous les cultivons sur le même milieu que précédemment, nous obtenons la même autodifférenciation (Pl. 1, fig. 1 et 2); alors que le soubassement de cette région, recouvert par d'autres ectoderme et mésoderme sous-ectodermique au stade indifférencié des germes plumaires défini par Sengel (1956), ne donne pas de morphogenèse glandulaire.

Ainsi, la couche superficielle du derme et l'ectoderme qui la recouvre (Fig. 1 dans le texte) sont, à ce stade, responsables de la formation des invaginations uropygiennes.

L'un de ces tissus a-t-il un rôle prépondérant dans l'induction de ces dépressions glandulaires? Cette question nous a incité à les séparer et à suivre leur évolution toujours sur le même milieu, soit isolément, soit en association avec d'autres tissus.

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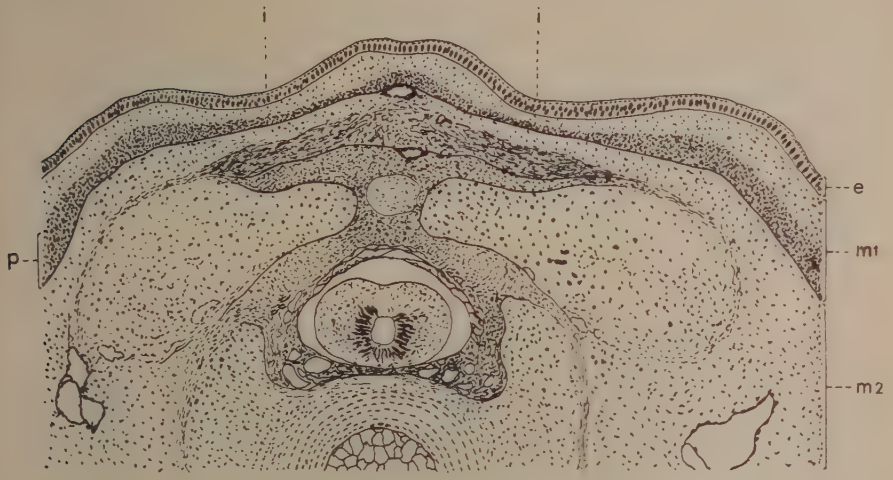

FIGURE 1. Dessin d'après une coupe transversale de la région caudale d'un embryon de Canard de 10½ jours au niveau des invaginations uropygiennes naissantes (i). *e*, ectoderme; *m1*, mésoderme sous-ectodermique qui se sépare du mésoderme profond *m2* sous la zone phosphatasique alcaline *p*.

MATÉRIEL ET TECHNIQUES

Nous avons employé des embryons de Canard de race Khaki Campbell, des embryons de Poulet de race Gâtinaise et accessoirement des embryons de Lapin, des larves de Batraciens et des alevins de Truite.

Toutes nos associations de tissus ont été cultivées sur le milieu standard de Wolff et Haffen (1952).

Les séparations de tissus ont été faites mécaniquement toutes les fois que cela a été possible d'une manière correcte, en particulier pour les séparations mésoderme-mésoderme et pour le décollement de l'ectoderme dorsal du bourgeon caudal, qui, à ces stades, adhère très peu au mésoderme sous-jacent et se prélève parfaitement à l'aide de pinces très fines (Pl. 2, fig. 2). Les fragments de mésoderme sous-ectodermique d'une région quelconque de l'embryon ont été obtenus par macération à 38° C au versène (Dihydrate du sel disodique de l'acide éthylène diamino-tétracétique) dans le liquide de Moscona selon la méthode employée par Zwilling (1955). Les fragments d'ectoderme ont été décollés, pour nos premières expériences, par macération à 38° C à la trypsine à 3 pour cent mélangée à l'hyaluronidase dans le liquide de Moscona (1952), toujours selon le procédé de Zwilling, puis par la suite par l'action de la trypsine seule à 0,5 pour cent dans le liquide de Moscona à 38° C pendant 5 à 10 minutes. En

dernier lieu, nous avons obtenu le meilleur résultat par la méthode de Szabó (1955) que nous avons adaptée aux tissus embryonnaires de la manière suivante: les fragments de peau sont épinglés dans une coupelle à fond de paraffine, et macérés de 18 à 24 heures dans une solution de trypsine Difco 1 : 250 à 0,005 pour cent dans du Tyrode à 4° C ou 2 heures dans une solution de trypsine Difco 1 : 250 à 0.3 pour cent à 4° C. L'ectoderme reste vivant (Pl. 2, fig. 1) et ne s'enroule pas lorsqu'on le déplace dans un liquide physiologique; à ces concentrations d'ailleurs, le mésoderme n'est pas très dissocié et peut aussi être utilisé comme explant.

RÉSULTATS EXPÉRIMENTAUX

Évolution des tissus du bourgeon caudal cultivés isolément

Bien que la partie sécrétrice de la glande uropygienne soit essentiellement de nature ectodermique, l'ectoderme de la région caudale, isolé de son support mésodermique et mis en culture, se rétracte fortement et donne une petite boule de cellules indifférenciées avec de gros grains de pigments noirs. Le mésoderme sous-ectodermique, qui se sépare du mésoderme profond sous la zone phosphatasique alcaline que nous avons mise en évidence dans un travail précédent (Gomot, 1956*a*), privé de sa couverture ectodermique et cultivé, prend un aspect granuleux blanchâtre et se nécrose au bout de quelques jours.

Ainsi, les deux constituants de l'explant ne donnent aucune morphogenèse lorsqu'ils sont cultivés séparément.

Évolution des associations de tissus prélevés avant l'apparition des invaginations uropygiennes

Le mésoderme caudal d'embryons de Canard de 10 jours, associé à de l'ectoderme quelconque ne recouvrant pas de germes plumaires individualisés, cultivé pendant 4 ou 5 jours, donne des invaginations ou des fentes épidermiques typiques (Pl. 1, fig. 3 et 4).

Mais l'ectoderme caudal d'embryons de 10 jours, transplanté sur du mésoderme sous-ectodermique quelconque du même âge au stade des ébauches dermiques des germes plumaires (Sengel, 1956), contribue à la formation de germes plumaires qui se développent très bien (Pl. 1, fig. 7 et Pl. 2, fig. 6). Ce même ectoderme caudal transplanté sur un mésoderme que nous appelons 'neutre' et qui n'est autre que le mésoderme pectoral profond (Pl. 2, fig. 7), ne donne aucune morphogenèse; l'ectoderme reste plat et se charge de gros grains de pigments.

De même si nous effectuons des changements d'orientation de 90° et 180° de l'ectoderme glandulaire sur son mésoderme, les invaginations se forment suivant l'orientation du mésoderme (Pl. 1, fig. 6).

Les mêmes résultats ont été obtenus avec des associations d'ectoderme et de mésoderme d'embryons de Poulets au 8ème jour de l'incubation (Pl. 1, fig. 10).

Donc la formation des invaginations uropygiennes est une interaction ectoderme-mésoderme où seul le mésoderme est spécifique.

Culture des associations de tissus précédentes faites lorsque les invaginations sont commencées

En cultivant les mêmes associations, faites lorsque les invaginations sont déjà commencées, les résultats changent progressivement au fur et à mesure que celles-ci s'approfondissent.

Au 11ème jour de l'incubation, si on recouvre le mésoderme des invaginations naissantes d'ectoderme quelconque, elles se continuent, s'approfondissent et se ferment normalement.

Mais l'ectoderme qui tapissait ces invaginations, transplanté sur un mésoderme neutre lâche, forme des plis très nets (Pl. 1, fig. 9) ou donne un réseau de petites fentes ectodermiques pigmentées (Pl. 1, fig. 8 et Pl. 2, fig. 8).

Au 12ème jour de l'incubation, les invaginations sont très profondes et on observe même au fond quelques plissements ectodermiques transversaux qui sont à l'origine des bourgeons primaires de la glande uropygienne. Néanmoins, le revêtement ectodermique s'enlève très facilement, mais, de par la forme de ces invaginations, maintenant profondes et étroites, l'ectoderme quelconque dont nous recouvrons le mésoderme caudal ne peut arriver à se mouler sur lui et au bout du deuxième jour de culture, le mésoderme s'est aplati et uniformisé sans qu'il y ait formation de glandes.

Quant à l'ectoderme de telles invaginations, sur un mésoderme neutre ou indifférencié, il se kératinise abondamment et s'exfolie en surface, mais il émet aussi à l'intérieur du mésoderme quelques bourgeons ectodermiques (Pl. 2, fig. 9) qui évoluent de la même manière et font penser à des tubes glandulaires uropygiens préfonctionnels ou à des germes plumaires tels que les ont obtenus Weiss & James (1955) en cultivant de la peau de Poulets Leghorns d'un âge correspondant.

Ces résultats nous amènent à penser que, lors de la phase primitive de la formation des invaginations uropygiennes, le mésoderme influence l'ectoderme qui le recouvre et induit, chez ce dernier, les nombreuses mitoses que l'on observe dans la zone des invaginations. Aussi, lorsqu'un tel ectoderme est transplanté sur un mésoderme neutre, ces mitoses se continuent encore un certain temps et provoquent la formation de plis ectodermiques (Pl. 2, fig. 8).

La formation des invaginations uropygiennes semble être comparable à la différenciation des germes plumaires étudiée par Sengel (1956) mais avec une polarité opposée, car, dans le cas des germes plumaires, il s'agit d'une évagination. Cependant, les plis ou les fentes ectodermiques obtenus par influence de l'ectoderme recouvrant de jeunes invaginations se sont estompés après 5 ou 6 jours de culture, alors que dans le cas des germes plumaires, ceux induits par un ectoderme similaire continuent à pousser.

Associations hétéroplastiques et xénoplastiques

Pour caractériser davantage cet organisateur glandulaire, nous avons encore réalisé quelques associations de tissus.

Si on interpose une couche de mésoderme quelconque indifférencié entre le mésoderme inducteur et l'ectoderme (Lutz & Gomot, 1956), il se forme tout de même des fentes ou des invaginations étroites (Pl. 1, fig. 5). L'action du mésoderme est donc capable de diffuser à travers une couche neutre.

Nous obtenons de même une morphogenèse glandulaire lorsque nous effectuons des associations hétéroplastiques de mésoderme caudal d'embryons de Canard et d'ectoderme d'embryons de Poulets Leghorns ou Gâtinais d'âge correspondant (Pl. 1, fig. 11) ou l'association inverse.

Ce résultat nous encourageant, nous avons tenté une série d'associations xénoplastiques de mésoderme inducteur avec de l'ectoderme d'embryon de Lapin et là encore nous avons obtenu la formation de petites invaginations ectodermiques (Pl. 1, fig. 12).

Mais lorsque nous avons recouvert le mésoderme inducteur par de l'ectoderme de têtard de Crapaud ou de Grenouille ou d'alevin de Truite, nous avons eu aussi un premier temps de morphogenèse glandulaire. Pendant les deux premiers jours de culture nous avons assisté à la formation rapide d'invaginations normales et profondes mais ensuite ces dernières étaient très vite remplies d'un tissu blanchâtre qui, en coupe, s'est révélé être du mésoderme sous-ectodermique. Ainsi après la formation d'invaginations profondes, les cellules du mésoderme inducteur traversent cet ectoderme étranger et comblent les dépressions glandulaires de telle manière, qu'en coupe, seule la membrane basale de l'ectoderme ajouté se reconnaît et marque encore la limite des invaginations qui s'étaient formées.

Finalement, nous avons recouvert le mésoderme inducteur par une 'membrane inerte' souple réalisée par de l'amnios ou de l'ectoderme d'embryon de Canard, tué par fixation à l'alcool absolu pendant 3 heures, puis lavé à l'eau distillée. L'évolution d'une telle association est analogue à celle de l'association précédente; les invaginations se forment puis la membrane est traversée par les cellules du mésoderme et les invaginations sont comblées.

DISCUSSION ET CONCLUSION

Si nous analysons la formation des invaginations glandulaires, dans le premier temps, le mésoderme seul semble édifier ces dépressions grâce à une multiplication cellulaire et des mouvements qui se font dans des directions bien déterminées, l'ectoderme jouant le rôle de 'membrane recouvrante'. Nous avons étudié les mouvements du mésoderme (Fig. 2 dans le texte) en suivant les déplacements de marques à l'encre de chine intercalées entre l'ectoderme et le mésoderme et en observant les changements de forme de la zone phosphatasique alcaline au cours de la formation des invaginations.

Après ce premier temps, pour que la morphogenèse glandulaire se poursuive, la couche recouvrante doit non seulement limiter les mouvements cellulaires du mésoderme mais réagir à son contact, sous l'action d'un inducteur qui diffuse, par de nombreuses mitoses qui permettent la formation des premiers bourgeons glandulaires.

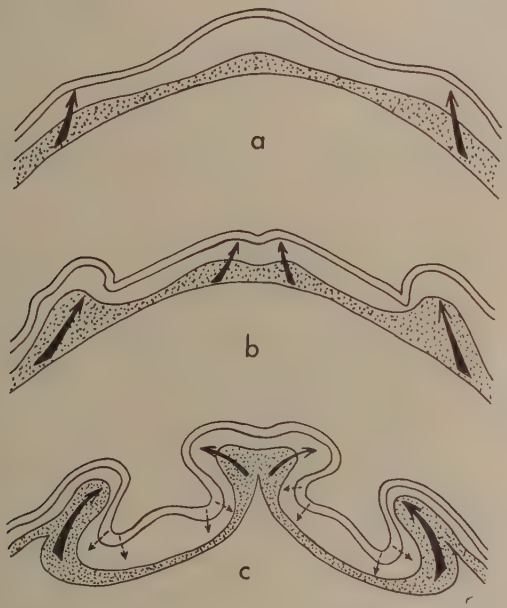

FIGURE 2. Schémas d'après des coupes transversales de la région uropygienne d'embryons de Canard de 10½ jours (Fig. 2*a*), 11 jours (Fig. 2*b*) et 12 jours (Fig. 2*c*). Nous avons matérialisé en traits pleins les principaux mouvements du mésoderme, en tirés les zones de multiplication intense de l'ectoderme où apparaissent les bourgeons glandulaires primaires et en pointillés la zone phosphatasique alcaline.

Ainsi en même temps qu'un travail mécanique de modelage, le mésoderme influence chimiquement l'ectoderme qui le recouvre de telle manière que ce dernier, transplanté à ce stade sur un mésoderme neutre, continue à se développer un certain temps conformément à la détermination que le mésoderme lui a communiquée. Nous avons en effet remarqué que ces mitoses ne sont pas réparties régulièrement dans tout l'ectoderme qui tapisse les invaginations mais qu'elles sont surtout nombreuses suivant certaines zones transversales qui donnent par la suite des plis ectodermiques sur le fond des invaginations, lesquels plis se fragmentent pour donner les tubes glandulaires primaires. Ces zones correspondent d'ailleurs à celles de moindre pression du mésoderme (Fig. 2*c* dans le texte).

La formation des invaginations uropygiennes résulte donc d'un travail synergique de l'ectoderme et du mésoderme sous-ectodermique caudaux. Le mésoderme donne la première impulsion mais elle doit être suivie d'une réaction bien définie de l'ectoderme qui le recouvre. Parmi les ectodermes que nous avons éprouvés, seuls ceux d'embryons d'Oiseau et de Mammifère se sont montrés capables d'une telle plasticité. L'ectoderme de larves de Batracien ou d'alevin de Poisson permet la formation des invaginations comme une membrane souple inerte mais, par la suite, il est très vite débordé par le mésoderme; il n'y a pas collaboration des tissus associés comme dans les cas précédents. Nous ne savons pas encore si cette incompatibilité est d'ordre sérologique ou si elle provient du fait que cet ectoderme d'animaux à 'sang froid' se trouve transplanté à une température plus élevée que celle du milieu où vivent les animaux donneurs.

RÉSUMÉ

1. La culture *in vitro* du bourgeon caudal d'embryons de Canard de 8, 9 et 10 jours permet la formation des invaginations uropygiennes qui apparaissent normalement au 11ème jour de l'incubation.

2. La séparation des divers tissus du bourgeon caudal d'embryons de Canard de 10 jours et leur culture en association avec d'autres tissus embryonnaires de Canard a permis de localiser, à ce stade, l'organisateur de la glande uropygienne dans la couche superficielle du derme.

3. La formation des invaginations glandulaires résulte d'une interaction ectoderme-mésoderme dans laquelle, à l'origine, seul le mésoderme est spécifique.

4. L'ectoderme d'embryons de Poulet et d'embryons de Lapin est capable de réagir au contact de ce mésoderme d'une manière analogue à de l'ectoderme d'embryon de Canard.

SUMMARY

1. The culture *in vitro* of the tail-bud of duck embryos aged 8, 9, and 10 days permits the formation of uropygial invaginations (rudiments of the preen gland) that normally appear on the 11th day of incubation.

2. The separation of the various tissues of the tail-bud of 10-day duck embryos and their culture in association with some other duck embryonic tissues has made it possible to locate, at this stage, the 'organizer' of the preen gland in the superficial stratum of the dermis.

3. The formation of the glandular invaginations results from an interaction between ectoderm and mesoderm, in which, at the beginning, only the mesoderm is specific.

4. The ectoderm of chick embryos and rabbit embryos is able to react to contact with this mesoderm in the same way as the ectoderm of duck embryo.

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EXPLICATION DES PLANCHES

PLANCHE 1

FIG. 1. Ectoderme et mésoderme sous-ectodermique dorsaux du bourgeon caudal d'un embryon de Canard au 8ème jour de l'incubation. A la mise en culture, l'explant était uniformément plat; après 3 jours de culture les germes plumaires apparaissent et du 4ème au 5ème jour il se forme deux petites fentes uropygiennes profondes bien individualisées.

FIG. 2. Explant identique au précédent mais provenant d'un embryon de 10 jours. Apparition d'invaginations qui se sont approfondies et commencent à se fermer normalement. Cultivé 4 jours.

FIG. 3. Bourgeon caudal d'un embryon de Canard de 10½ jours. Son ectoderme a été remplacé par de l'ectoderme dorsal obtenu mécaniquement. Les invaginations sont déjà en partie refermées dans la région antérieure. Cultivé 4 jours.

FIG. 4. Association identique à la précédente mais l'ectoderme provient de l'aile et a été séparé par action de la trypsine. Cultivée 3 jours.

FIG. 5. Intercalation d'une couche de mésoderme indifférencié entre le mésoderme caudal d'un embryon de 10½ jours et de l'ectoderme quelconque. Formation de fentes épidermiques.

FIG. 6. Ectoderme du bourgeon caudal d'un embryon de Canard de 10 jours tourné de 180° sur son support mésodermique. Les invaginations se forment suivant l'orientation du mésoderme.

FIG. 7. Mésoderme sous-ectodermique du cou d'un embryon de Canard au 10ème jour de l'incubation. Recouvert par l'ectoderme caudal d'un embryon de 10 jours, il contribue à la formation de germes plumaires. Cultivé 4 jours.

FIG. 8. Mésoderme pectoral 'neutre' recouvert par l'ectoderme d'invaginations uropygiennes, il se forme des fentes ectodermiques profondes. Cultivé 4 jours.

FIG. 9. Mésoderme pectoral 'neutre' recouvert par l'ectoderme des invaginations uropygiennes d'un embryon de Canard de 11 jours. Après 2 jours de culture, la surface est toute plissée.

FIG. 10. Derme caudal superficiel d'un embryon de Poulet de 8½ jours associé à de l'ectoderme de l'aile du même embryon. Les invaginations sont fermées et le mamelon, plus développé que chez le Canard, est nettement individualisé.

FIG. 11. Association du mésoderme caudal d'un embryon de Canard de 10½ jours avec de l'ectoderme d'embryon de Poulet de 7 jours. Cultivée pendant 3 jours, elle donne naissance à des invaginations uropygiennes typiques.

FIG. 12. Association du mésoderme caudal d'un embryon de Canard de 10 jours avec de l'ectoderme d'embryon de Lapin. Pendant les deux premiers jours de culture, l'ectoderme semble se nécroser, puis il y a formation de deux invaginations en croissant qui se referment et laissent deux fentes étroites.

Planche 2

Fig. 1. Ectoderme du cou d'un embryon de Canard au 10ème jour de l'incubation obtenu par macération à la trypsine. Grossissement: ×850.

Fig. 2. Ectoderme caudal dorsal d'un embryon de Canard de 10 jours prélevé mécaniquement. Grossissement: ×850.

Fig. 3. Coupe transversale au niveau des invaginations de l'explant de Pl. 1, fig. 2. Grossissement: ×70.

Fig. 4. Coupe transversale d'une association de mésoderme caudal avec de l'ectoderme dorsal quelconque. Après 5 jours de culture les invaginations sont fermées et les bourgeons primaires commencent à se développer. Grossissement: ×110.

Fig. 5. Coupe transversale au niveau des fentes de l'explant de Pl. 1, fig. 5; *mi*, mésoderme intermédiaire. Grossissement: ×290.

Fig. 6. Coupe transversale de l'explant de Pl. 1, fig. 7. Grossissement: ×94.

Fig. 7. Coupe transversale de la région pectorale d'un embryon de Canard de 11 jours. *e*, ectoderme; *cm*, zone des concentrations mésodermiques correspondant aux futurs germes plumaires; *mn*, mésoderme 'neutre'. Grossissement: ×134.

Fig. 8. Coupe transversale au niveau de 2 fentes de l'explant de Pl. 1, fig. 8. Grossissement: ×370.

Fig. 9. L'ectoderme recouvrant les invaginations uropygiennes d'un embryon de Canard de 12 jours, transplanté sur du mésoderme pectoral profond, s'exfolie en surface mais donne aussi des bourgeons ectodermiques dont les cellules centrales prennent une structure en bulbe de lys et dégénèrent. Coupe transversale. Grossissement: ×250.

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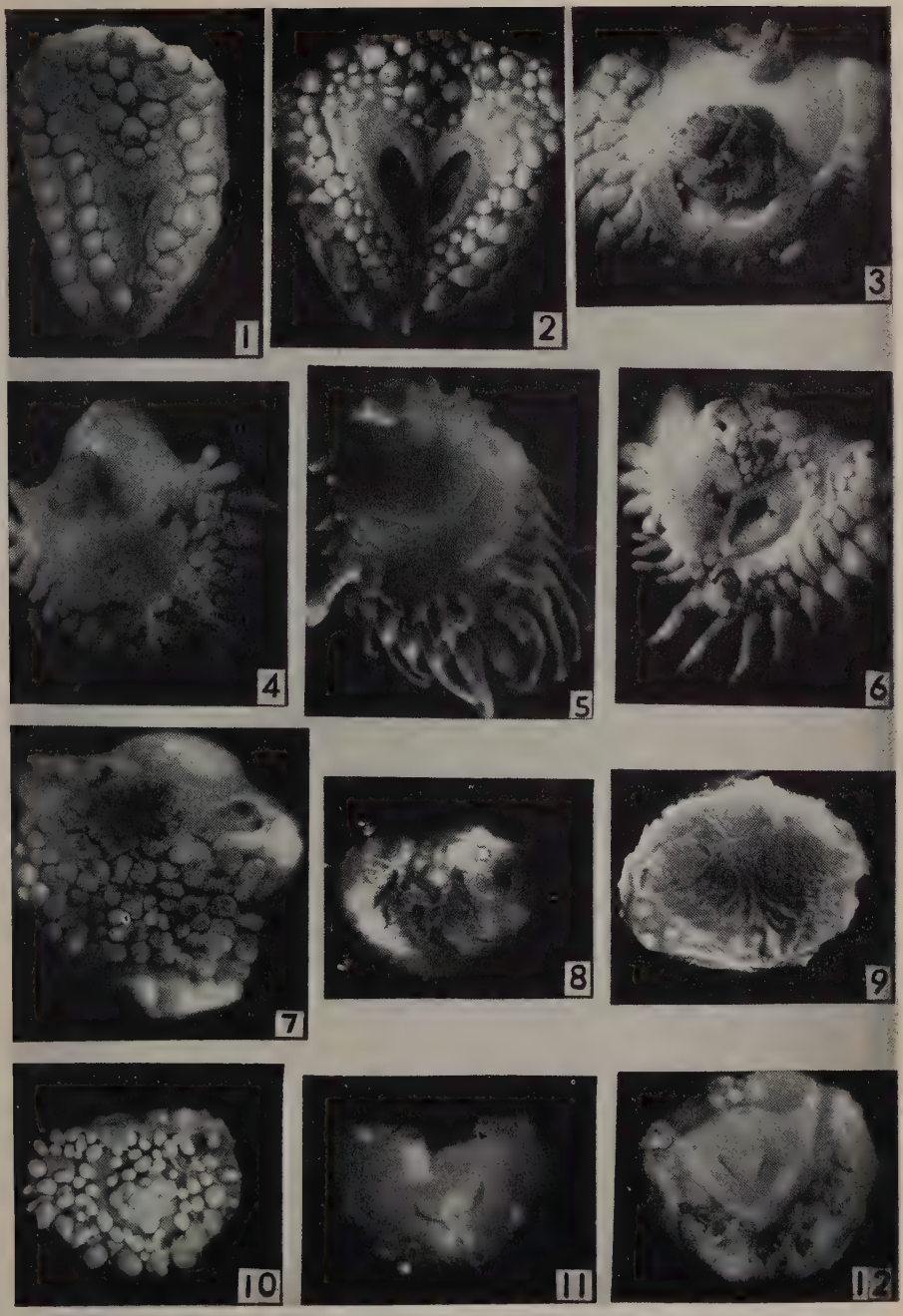
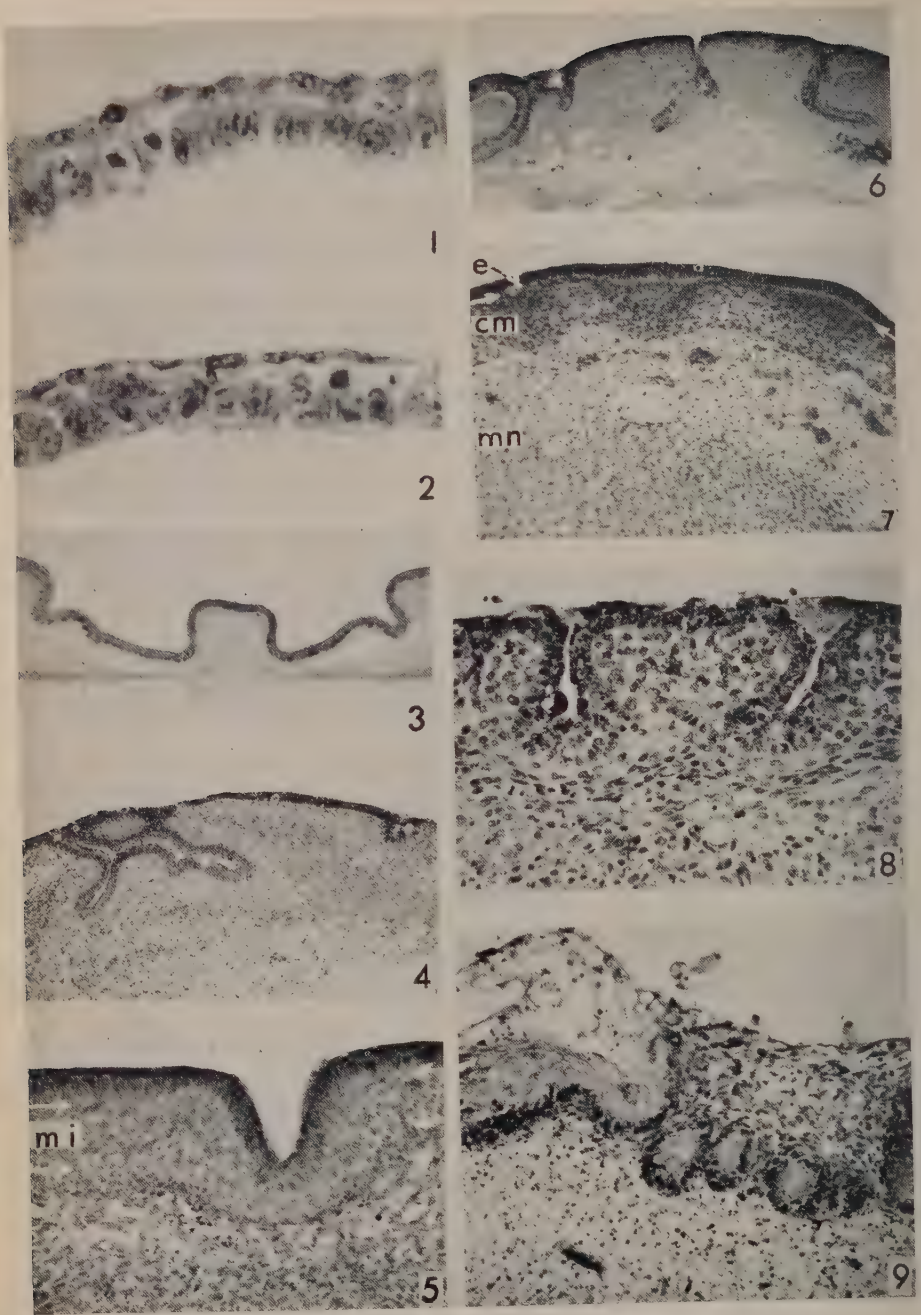


L. GOMOT

Plate 1



L. GOMOT

Plate 2

Physiologie du cœur de l'embryon de Poulet *in vitro* après congélation à très basse température

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La découverte (Rostand, 1946; Polge, Smith, & Parkes, 1949) des remarquables propriétés protectrices de la glycérine a permis de soumettre à des froids très intenses, divers tissus et organes, tout en leur conservant un degré de survie important. Nous avons voulu analyser sur un exemple précis quelles étaient les conditions de protection qu'offrait le traitement par la glycérine.

TECHNIQUE

Matériel expérimental

Nous avons utilisé le cœur de l'embryon de Poulet (Leghorn blanc) au bout de 7 jours $\frac{1}{2}$ d'incubation. Ce cœur, après dissection stérile, est cultivé en culture d'organes selon la technique du professeur Wolff (Wolff & Haffen, 1952) sur milieu solide (solution physiologique d'Agar, extrait embryonnaire, solution physiologique tamponnée). Les cultures sont incubées à la température de 37° C pendant 48 heures, et deux examens sont faits au bout de 24 et de 48 heures. Le test d'activité physiologique utilisé a été les battements des oreillettes et des ventricules.

Techniques de congélation

Les cœurs ont été congelés, soit directement, soit après imprégnation pendant 30 minutes dans une solution physiologique (liquide d'Earle) contenant divers pourcentages de glycérine. Les cœurs témoins sont conservés pendant le même temps dans la solution physiologique pure, à + 20° C.

Trois techniques de congélation différentes sont utilisées.

(1) *Congélation ultra-rapide.* Les cœurs sont montés sur des rubans d'aluminium et immergés brutalement dans du propane liquide maintenu à la température de l'azote liquide (− 196° C). L'utilisation de propane permet une congélation extrêmement rapide par suite de l'absence de phénomènes d'ébullition et de caléfaction autour du fragment. Nous avons pu montrer par des

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expériences de cultures de tissus préalables (Rey, 1957) qu'un séjour de 5 à 30 secondes dans le propane liquide ne provoquait pas d'altérations. La durée de congélation d'un cœur entier, mesurée à l'oscillographe cathodique, est de l'ordre de 0,5 seconde (Fig. 1). Les cœurs sont laissés 15 secondes dans le propane liquide, puis transférés dans l'azote liquide pendant 30 minutes.

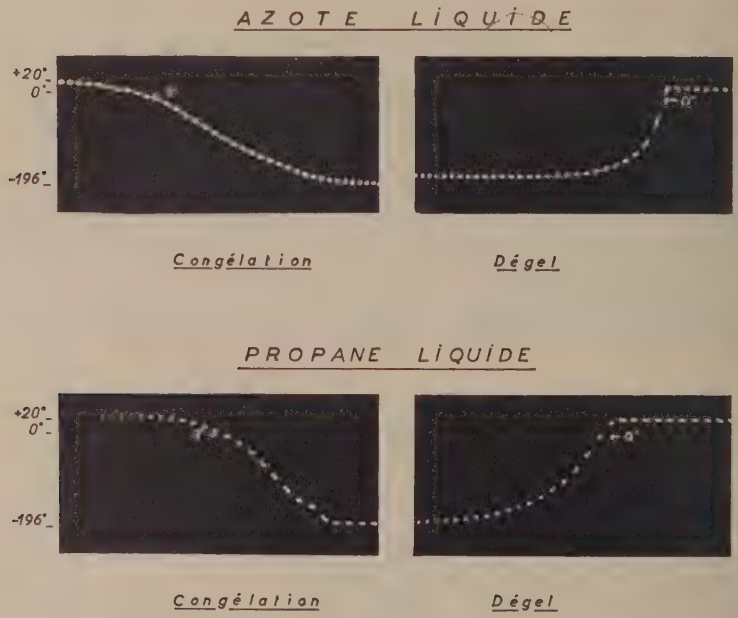

FIG. 1. *Évaluation de la vitesse de refroidissement des cœurs à très basse température*

Congélation directe du cœur dans l'azote liquide et dans le propane liquide à −196° C. Dégel direct dans une solution physiologique à +20° C. Couple cuivre-constantan de 0,6 mm. Enregistrement à l'oscillographe cathodique avec repérage du temps par modulation basse-fréquence. Chaque trait représente $\frac{1}{20}$ de seconde.

(2) *Congélation rapide.* Une technique semblable est utilisée, mais les rubans d'aluminium sont plongés dans l'azote liquide directement. Le temps de congélation est de l'ordre de 1,5 secondes (Fig. 1).

(3) *Congélation lente.* (*a*) *Congélation directe.* Les cœurs sont montés sur une lamelle de verre de 4/10 mm d'épaisseur, placée dans un tube de pyrex de 11 mm de diamètre et de 1 mm de paroi. Ce tube est immergé dans l'azote liquide. Le temps de congélation est de l'ordre de 1 minute.

(*b*) *Congélation par étapes.* Pour congeler à des températures intermédiaires, entre 0° C et l'azote liquide, nous avons utilisé l'enceinte thermostatique pour basses températures que nous avons décrite précédemment (Rey, 1957). Les

cœurs contenus dans des tubes de pyrex, comme précédemment, sont congelés d'abord à − 30° C ou à − 60° C, puis ensuite plongés dans l'azote liquide.

Techniques de dégel

Pour étudier l'influence du dégel, nous avons dégelé les préparations après congélation, selon 3 vitesses différentes.

(1) *Dégel très rapide.* Les rubans d'aluminium ou les lamelles de verre sont immergés directement dans une solution physiologique à + 20° C. Divers essais ont été effectués en utilisant, soit une solution physiologique simple, soit une solution physiologique contenant de la glycérine. Le temps de dégel est de l'ordre de 0,5 seconde (Fig. 1).

(2) *Dégel à vitesse moyenne.* Le tube de pyrex contenant les explants est plongé dans de l'eau à + 20° C. Le dégel est complet au bout de 45 secondes.

(3) *Dégel très lent.* On laisse réchauffer le tube à l'air à + 20° C. Il faut environ 6 minutes pour que le dégel soit complet.

Évaluation de l'activité physiologique

Des expériences préliminaires nous ont montré, qu'après congélation, le tissu cardiaque conservait une grande partie de sa viabilité lorsqu'il avait été imprégné au préalable par une concentration adéquate de glycérine. Ce degré de survie a été apprécié par la culture *in vitro* de fragments du cœur congelé. Dans cette deuxième série d'expériences, nous avons voulu voir comment était conservée l'activité fonctionnelle du cœur dans son ensemble. Nous avons utilisé deux tests principaux.

(1) *L'examen des battements cardiaques.* Selon le degré de survie, l'intensité des battements est plus ou moins grande et nous avons défini une échelle d'activité croissante: aucun battement — battements de points ventriculaires isolés — battements d'un ventricule unique — battements des deux ventricules — battements des oreillettes et des ventricules — et battements coordonnés des oreillettes et des ventricules, ce dernier stade représentant l'activité physiologique maximum.

Nous avons étudié la variation de la fréquence des battements en fonction de la température entre + 32° C et + 38° C.

(2) *L'évolution du pH du milieu de culture* nous permet d'évaluer, de façon approchée, l'activité métabolique du cœur pendant la période précédant l'examen. Pour cela, nous avons incorporé à la solution physiologique du milieu de culture, des traces de rouge de phénol. Cet indicateur utilisé couramment en cultures de tissus, n'est en effet pas toxique. Dans les cultures où le cœur a conservé une activité physiologique importante, le pH évolue très rapidement vers l'acidité.

RÉSULTATS EXPÉRIMENTAUX

Comportement des témoins

Les cœurs témoins en culture conservent des battements réguliers pendant 48 heures environ. Il s'agit de battements simultanés des oreillettes et de batte-

ments simultanés des ventricules, avec, le plus souvent, coordination des mouvements auriculo-ventriculaires. La fréquence des battements dépend de la température ambiante et, en première approximation, entre 32° et 38° C, cette variation est linéaire. La fréquence propre varie assez sensiblement d'un cœur à l'autre, mais le coefficient de température est exactement le même. L'examen histologique montre qu'au bout de 48 heures l'organe est encore parfaitement conservé et il n'y a que peu de nécrose à l'intérieur. Au bout de 24 heures de culture, par contre, la nécrose est pratiquement inapparente, et c'est généralement la période que nous choisissons pour tester l'activité physiologique.

Comportement des cœurs congelés

(1) *Influence de la glycérine.* Nous avons trouvé des résultats identiques à ceux que nous avons obtenus par la méthode de cultures de tissus. L'imprégna-

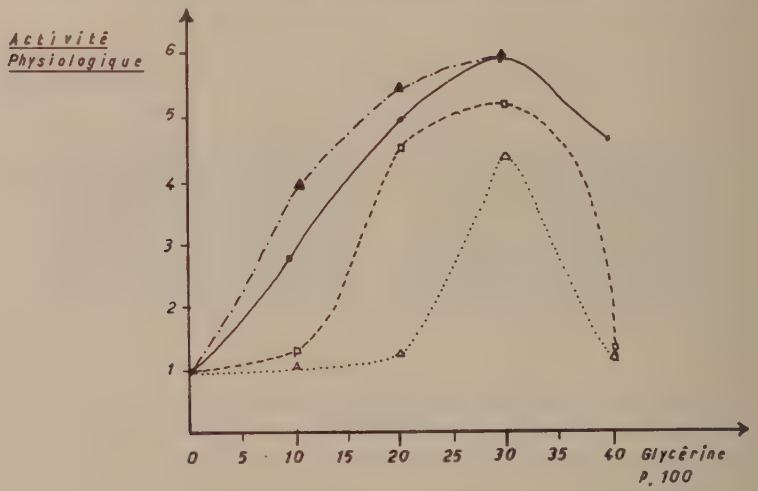

FIG. 2. *Variation de l'activité physiologique en fonction des vitesses de congélation*
—— Congélation en tube dans l'azote liquide (65 secondes).
-----Congélation directe dans le propane liquide (0,5 seconde).
.....Congélation directe dans l'azote liquide à −196° C (1,5 seconde).
–.–.– Congélation en tube à −30° C (40 secondes) puis à −196° C (20 secondes).

L'activité physiologique comporte 6 niveaux différents:
1 aucune activité physiologique.
2 battements de points ventriculaires isolés.
3 battements de 1 ventricule unique.
4 battements des 2 ventricules.
5 battements des oreillettes et des ventricules.
6 battements coordonnés des oreillettes et des ventricules.

tion préalable par une solution physiologique contenant un certain pourcentage de glycérine est nécessaire au maintien de la viabilité. Quelles que soient les vitesses de congélation et de dégel, aucun cœur non imprégné n'a pu survivre.

Il existe un optimum dans la concentration de glycérine qui est de 30 pour cent en poids.

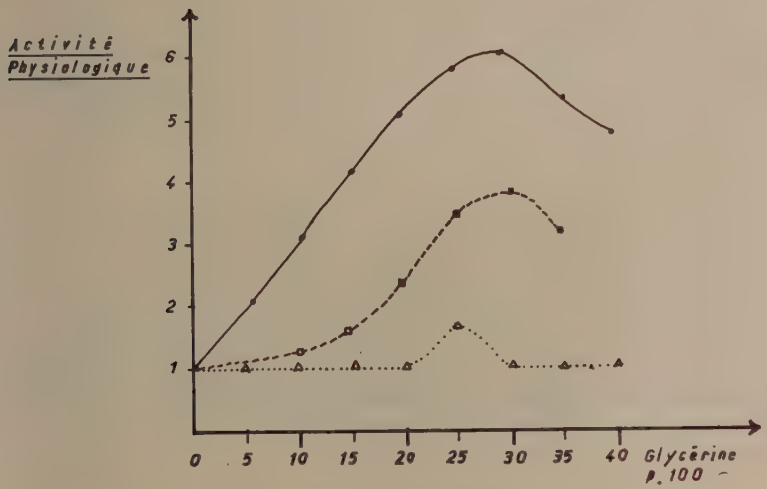

FIG. 3. *Variation de l'activité physiologique en fonction des vitesses de dégel*
—— Dégel rapide à +20° C (1 seconde).
----- Dégel lent en tube (45 secondes).
..... Dégel très lent à l'air (6 minutes).
Activité physiologique: même notation que pour la Fig. 2.

Des expériences de contrôle nous ont montré, que même après une ou deux heures de séjour dans la solution glycérinée, il n'y avait aucune action toxique apparente.

(2) *Influence de la vitesse de congélation.* La figure 2 indique que pour une concentration optima de glycérine (30 pour cent en poids), l'activité physiologique varie relativement peu avec la vitesse de congélation. Toutefois, pour des vitesses extrêmement élevées (propane liquide direct et azote liquide direct), il semble qu'il y ait une légère diminution de l'activité physiologique. Nous avons pu relier cette diminution au fait que, lors de ces congélations extrêmement rapides, il se produit des craquelures et des lésions anatomiques importantes du cœur. Par contre, pour les concentrations de glycérine inférieures ou supérieures à l'optimum, la congélation diminue considérablement l'activité physiologique, et il semble alors que les meilleurs résultats soient obtenus par une congélation lente procédant par étapes.

(3) *Influence des vitesses de dégel.* Le plus souvent, il semble que l'on n'ait pas accordé beaucoup d'intérêt aux vitesses de dégel. Cependant, une très grande partie des altérations provoquées par le froid est due à de mauvaises conditions de dégel. Les résultats que nous avons obtenus montrent qu'après congélation à une vitesse moyenne (représentant l'optimum), il est essentiel

que le dégel soit le plus rapide possible (Fig. 3). Les dégels lents ou très lents provoquent des altérations très graves, et toute viabilité disparaît. Nous avons trouvé des résultats pratiquement identiques lorsque le dégel est effectué dans une solution physiologique à + 20° C ou à une température plus élevée (+ 39° C). Lorsque la solution utilisée pour le dégel est la même que celle ayant servi à l'imprégnation, les résultats sont les meilleurs. Par contre, aucune amélioration n'est apportée par un dégel effectué dans une solution physiologique simple ou contenant des concentrations de glycérine plus élevées ou plus faibles.

(4) *Conservation de longue durée.* Nous avons essayé, après congélation, de conserver les cœurs à basse température pendant plusieurs semaines.

Une première série d'expériences nous a montré qu'à la température de - 80° C (glace carbonique), la viabilité était parfaitement maintenue, et qu'au bout de quelques semaines, après un dégel rapide, le cœur reprenait une activité physiologique presque normale. Pour connaître exactement à quelle température commencent à apparaître les premiers signes d'altération, nous avons entrepris des recherches nouvelles dans lesquelles nous conservons des cœurs congelés, à des températures différentes maintenues soigneusement constantes. Nous donnerons les résultats ultérieurement.

CONCLUSION

L'analyse de la protection offerte par la glycérine contre les effets toxiques du séjour aux basses températures, nous a montré qu'il existait un optimum qui se situe à 30 pour cent en poids pour le cœur de l'embryon de poulet. Après imprégnation par la solution glycérinée et congélation, il ne semble pas que l'activité physiologique soit modifiée par le froid. En effet, non seulement les cœurs ont un comportement physiologique normal avec un coefficient thermique identique à celui des témoins, mais de plus il n'y a pas de signes physiologiques ou histologiques d'altérations.

Il est encore difficile d'expliquer de façon précise comment les solutions glycérinées inférieures à l'optimum, apportent une protection partielle. Il semble que les différents niveaux d'activité physiologique soient dus au maintien à l'état vivant d'un nombre plus ou moins grand de cellules. Il reste encore à expliquer pourquoi certaines cellules sont protégées et pourquoi d'autres, au contraire, sont détruites. Plusieurs explications de ce phénomène sont possibles. On peut, d'une part, envisager qu'il existe dans un même groupe cellulaire des degrés de résistance au froid variables, et que certaines lignées cellulaires sont moins sensibles que d'autres; on peut, d'autre part, penser que l'action différentielle de la congélation est due à une répartition inégale des processus de cristallisation et que certaines cellules cristallisent de façon plus brutale que d'autres et se trouvent ainsi mortellement lésées.

De toute manière, pour les faibles concentrations en glycérine, il semble que les cellules survivantes soient atteintes de façon sérieuse. En effet, en culture

de tissus, nous avons pu montrer que pour des concentrations en glycérine inférieures à 5 pour cent il y avait une certaine migration cellulaire après la mise en culture, mais que ces cellules n'étaient pas susceptibles d'une survie prolongée et manifestaient, au bout de 48 heures, des phénomènes de dégénérescence graisseuse avec apparition de très grosses vacuoles huileuses dans le cytoplasme. Une constatation identique a été faite par McPherson, Draheim, Evans, & Earle (1956) dans les cultures de cornée.

RÉSUMÉ

L'activité physiologique du cœur de l'embryon de Poulet est étudiée *in vitro* après exposition préalable à de très basses températures. La glycérine exerce une action protectrice qui varie avec son pourcentage et l'optimum est atteint par 30 pour 100 en poids. L'étude précise des conditions de refroidissement et de réchauffement montre que le dégel constitue une phase critique et qu'il doit être le plus rapide possible. L'auteur suggère quelques hypothèses expliquant la protection partielle apportée par des solutions glycérinées différentes de l'optimum.

SUMMARY

The physiological activity of the chick embryo heart after an initial exposure to very low temperatures has been studied *in vitro*. Glycerine has a protective action which varies with its concentration, the optimum being 30 per cent. by weight. A precise study of the conditions of cooling and of rewarming show that thawing is a critical phase and must be as rapid as possible. Certain hypotheses are suggested to explain the partial protection provided by solutions of glycerine differing from the optimum.

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In vitro Culture of the Eye and the Retina of the Mouse and Rat

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WITH TWO PLATES

INTRODUCTION

STRANGEWAYS & FELL (1926) and Dorris (1938) showed that embryonic chick eyes could be cultivated successfully *in vitro* on plasma clots, and Harrison (1951) and Reinbold (1954) obtained satisfactory results with an agar-tyrode medium containing embryo extract. The retina in these eyes was undifferentiated and, though little or no increase in the size of the eyes occurred, the authors agreed that cytological differentiation proceeded at a more or less normal rate.

Tansley (1933) reported the successful cultivation on plasma clots of whole eyes from rat embryos of 14–17 days and of isolated retina from rats up to 5 days old. Cytological differentiation proceeded at a normal rate, at least for the first few days in culture, but excessive growth of mesodermal elements tended to occur after 12 to 14 days. Rosette formation was a prominent feature in these cultures.

The present work differs from previous studies in that most of the material cultured was more mature and a fluid medium free from embryo extract has been used. The maturity of the material was governed chiefly by the fact that it is at this stage of retinal differentiation that retinal dystrophies, which will be the subject of a separate study, occur (Sorsby *et al.*, 1954; Lucas *et al.*, 1955).

MATERIAL AND METHODS

Material

CBA mice aged 8–14 days. Albino rats aged 1–2 and 9–14 days. Both lines are maintained on a cubed diet at the M.R.C. Radiobiological Unit, Harwell.

The animals were decapitated and the eyes removed aseptically. In the case of retinal cultures, the retina was dissected out in culture fluid. In mice the retina was divided into two pieces, and in rats it was divided into four.

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Culture technique

The culture chamber was made of Perspex (Lucite) and contained a shallow dish in which was a sheet of lens paper supported on a tantalum wire grid exactly as described by Trowell (1954). The culture medium was the synthetic medium (TACPI) described by Trowell (1955) with the addition of 15 per cent. rat serum. The volume of culture medium was 6 ml. and the medium was changed after 4 days. The chambers were either circulated very slowly with a mixture of 97 per cent. oxygen and 3 per cent. CO_2 or left containing air. When the chambers contained air the side tubes were sealed off, and the pH of the culture medium was controlled by periodically opening one of the side tubes to allow escape of CO_2.

Histological technique

The tissues were fixed in acid Zenker's fluid, dehydrated in cellosolve, and embedded in ester wax (Chesterman & Leach, 1956). Sections were cut at 5 μ and stained with Mayer's haemalum and alcoholic eosin.

RESULTS

Whole eyes

Some excellent results were obtained with the eyes of mice 8–11 days old, cultured in 97 per cent. oxygen with the cornea uppermost. In some of these all layers of the retina survived in good condition for up to 8 days (Plate 1, figs. A, B). Limited areas of necrosis were, however, sometimes present at the posterior pole. There was some folding and tunnel formation in the outer nuclear layer, but, if the eye was not punctured when planted, no herniation of retina through the sclera occurred as reported by Strangeways & Fell (1926) and Tansley (1933). The deformation of the outer nuclear layer made it difficult to ascertain whether any cells had been lost, but any such loss was certainly less than in the isolated retina (see below).

In the eyes of rats, aged 1–2 days, cultured in air or oxygen for 4–8 days, most of the retina became necrotic whichever way up the eye was placed. Such areas of retina as survived usually showed some structural disorganization.

The other ocular tissues, including lacrimal gland, survived well for the period of cultivation whether under 97 per cent. oxygen or air. The centre of the lens underwent necrosis, but the epithelium and adjacent lens fibres remained intact.

Isolated retina

The retina became doubled over during dissection and division into two or four pieces and, during culture, the lower layer usually degenerated, but the upper layer survived remarkably well. Retinae of both rats and mice of age 9–14 days cultured in air for up to 10 days—the longest period tried—showed good histological preservation (Plate 1, fig. D).

The number of cells in both inner and outer nuclear layers was reduced in the cultured retinae by up to one-third as compared with *in vivo* controls. The thickness of the inner fibre layers, which normally is increasing at this age, was also less than in controls (Plate 1, fig. C). In some mouse retinae the rod nuclei were apparently being replaced by nuclei with a much more open chromatin pattern resembling cone nuclei, but more likely to be glial elements. Mitotic figures were very rarely seen.

Higher concentrations of oxygen were found to be toxic. Retinae cultured in 97 per cent. or even 60 per cent. oxygen were totally necrotic after a few days, though the ciliary epithelium (pars ciliaris retinae) survived satisfactorily (Plate 2, fig. H). The ciliary epithelium arises from the anterior part of the retina during post-natal development and it is interesting that this epithelium should be resistant to high concentrations of oxygen, whereas all the elements of the retina proper were completely destroyed.

In an entirely synthetic medium (TACPI) rat retinae survived quite well for 7 days (Plate 2, fig. I), but mouse retinae did rather poorly (Plate 2, fig. J).

Differentiation of visual cells

In rats and mice aged 9–11 days, the nuclei of the visual cells are ovoid (Plate 2, fig. E), and the outer segments only just evident. During the ensuing few days rapid elongation of the rod elements normally occurs (Detwiler, 1932) and slower differentiation of the nuclei (Sorsby *et al.*, 1954; Lucas *et al.*, 1955). The nuclear change consists of coalescence of the chromatin, first to two or more large masses (Plate 2, fig. F), and eventually, after several weeks, to a single spherical mass surrounded by a delicate crenated membrane. In the cultures this nuclear differentiation appeared to proceed exactly as *in vivo* (Plate 2, fig. G), although no further growth of the rod elements occurred.

DISCUSSION

The advantages of this culture method have already been discussed (Trowell, 1954) and the present results show that by this technique the retina of the rat and mouse can be maintained in good histological condition for about 10 days. Under these conditions, however, the fibre layers and sensory organelles do not grow further. The loss of nuclei from the nuclear layers may well be due to loss of cells from trauma during dissection. Retinae examined shortly after dissection showed a certain number of pycnotic nuclei.

The adverse effect of 97 per cent. oxygen on the isolated retina is one of the most interesting features of the present work. In the case of the whole-eye cultures, the outer layers would consume oxygen and also hinder its diffusion to the retina, so the better survival of the retina when 97 per cent. oxygen was used was not unexpected. The somewhat variable results obtained when the eye was placed cornea uppermost may well have been due to the difficulty of achieving

a satisfactory balance between oxygen-deprivation and oxygen-poisoning over the whole retinal field. The poor results obtained when the eye was placed cornea downwards are likely to have been due to oxygen-poisoning, since the anterior part of the retina was often spared.

Other workers have shown that the retina, and also the cerebral cortex, are peculiarly susceptible to oxygen poisoning. Thus, Gyllensten & Hellström (1955) kept 10-day-old mice in an atmosphere of oxygen and found that this caused atrophy of the inner retinal layers. Noell (1955) kept adult rabbits in an atmosphere of oxygen and found widespread necrosis of the visual cells within a few days. Dickens (1946) incubated brain slices in Warburg manometers, using a gas phase of pure oxygen, and found that respiration was slowly and irreversibly poisoned after a few hours.

One of us (Trowell, to be published) has found that the following organs of the young rat can be successfully cultured for at least a week, using the technique described here and a gas phase of 97 per cent. oxygen and 3 per cent. CO_2: lymph node, thyroid, parathyroid, pituitary, pineal, ovary, uterus, fallopian tube, vas deferens, prostrate, ureter, trachea, lung, adipose tissue, submandibular gland, and sympathetic ganglion. For all these organs culture in oxygen gave more satisfactory results than culture in air, and there was no sign of oxygen-poisoning. The retina is the only exception which we have so far encountered.

Strain C3H mice exhibit a genetically determined retinal dystrophy which begins to appear at age 11 days. The results of culturing potentially dystrophic C3H retinae will be reported in a subsequent paper.

SUMMARY

1. By means of an organ culture technique previously described, whole eyes of 8–11-day-old mice have been successfully maintained *in vitro* for 8 days. Isolated retinae from both rats and mice aged 9–14 days have been similarly maintained for 10 days.

2. Isolated retinae survived best in a gas phase of air; 60 per cent. oxygen was toxic. In the whole eye, on the other hand, the retina survived better when pure oxygen was used, doubtless because the outer coats of the eye both consume oxygen and hinder its diffusion to the retina.

3. Isolated rat retinae survived well in an entirely synthetic medium, but mouse retinae did not.

4. Differentiation of the visual cell nuclei proceeded normally *in vitro*.

ACKNOWLEDGEMENT

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EXPLANATION OF PLATES

Plate 1

Fig. A. Eye of mouse aged 11 days after 7 days' culture in 97 per cent. oxygen. ×15.

Fig. B. Retina of the eye shown in fig. A. ×335.

Fig. C. Retina of mouse aged 21 days. For comparison with B and D. ×335.

Fig. D. Retina of mouse aged 11 days after 10 days' culture in air. A double layer was cultured, the upper layer has survived well, the under layer is atrophied and entangled with lens paper. ×335.

Plate 2

Fig. E. Visual cell nuclei of mouse aged 11 days. ×1,840.

Fig. F. Visual cell nuclei of mouse aged 21 days. ×1,840.

Fig. G. Visual cell nuclei of retina of mouse aged 11 days after 10 days' culture in air. Compare with E and F. ×1,840.

Fig. H. Retina of rat aged 13 days after 7 days' culture in 60 per cent. oxygen. All elements of the neuroretina are dead but the ciliary epithelium is healthy. ×335.

Fig. I. Retina of rat aged 13 days after 7 days' culture in synthetic medium in air. ×335.

Fig. J. Retina of mouse aged 10 days after 7 days' culture in synthetic medium in air. ×335.

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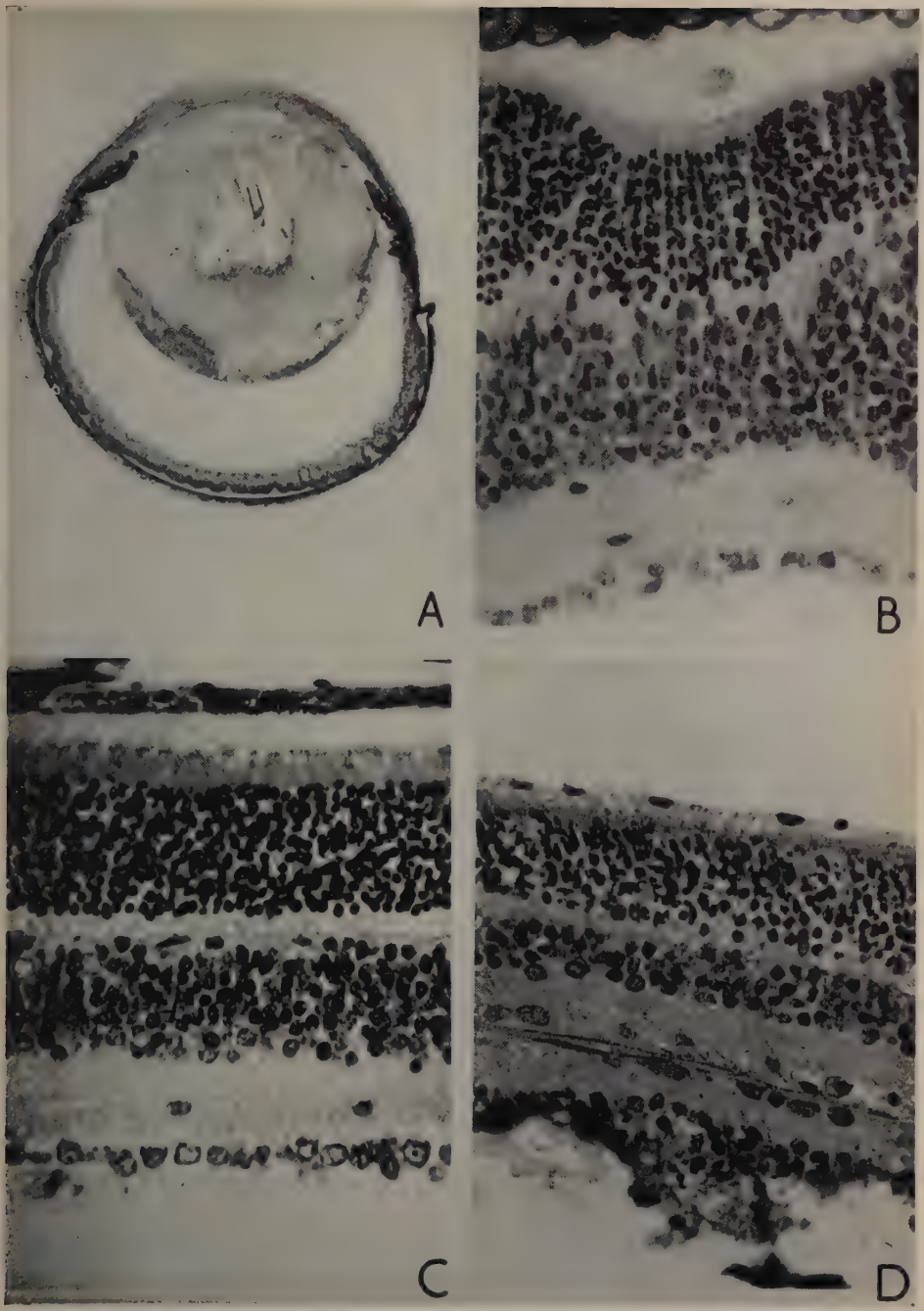

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Plate 1

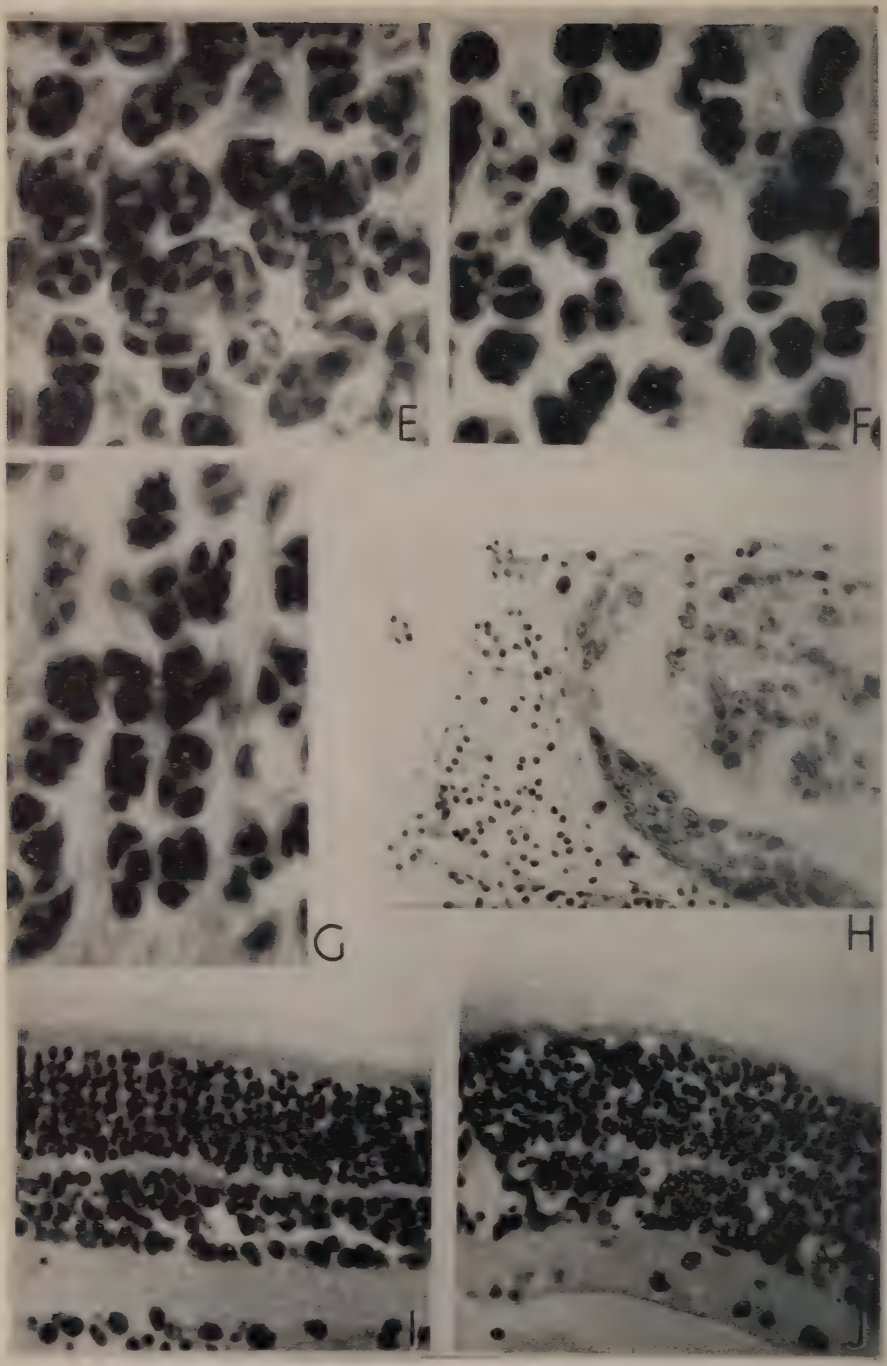

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Plate 2

INSTRUCTIONS FOR CONTRIBUTORS

Contributions and correspondence about them should be sent to Mr. M. Abercrombie, Department of Anatomy and Embryology, University College, Gower Street, London, W.C. 1, England.

Contributions should be as concise as possible. They should be typewritten, double-spaced, on one side of the paper, the pages numbered. They should be fully ready for press, since revision in proof will not be possible. Footnotes should be avoided. The author is advised to keep a copy of the typescript.

Tables should be on separate sheets, and numbered. Authors should indicate the places for their insertion in the margin of the typescript.

The Summary should be in numbered paragraphs, and will be put at the end of the text. It should not exceed 500 words.

Acknowledgements should be placed immediately before the list of references.

References should be listed alphabetically. In the text they should be referred to by the author's name and the year of publication. If more than one reference to the same author and year is quoted, use suffixes *a*, *b*, etc. (e.g. 1951*a*) in text and reference list. Papers should be listed as follows: Surname followed by initials of author(s); year of publication; title of paper; name of Journal (World List of Scientific Periodicals abbreviation), underlined; volume number; first and last page: e.g. Spemann, H., & Mangold, H. (1924). Über Induktion von Embryonalanlagen durch Implantation artfremder Organisatoren. Arch. mikr. Anat. **100**, 599–683. Books should be listed as follows: Surname followed by initials of author(s); year of publication; title underlined; place of publication; publisher: e.g. Dalcq, A. (1941). L'Œuf et son dynamisme organisateur. Paris: Albin Michel.

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Black and white line or line and dot drawings should have all lettering or numerals put in lightly in block capitals with a soft pencil. Charts and graphs may be drawn in Indian ink on graph paper ruled in pale blue. Where several drawings are grouped as one figure, distinguish them by capital letters (in pencil). Legends for all line drawings should be typed together on a separate sheet. The places for insertion in the text should be indicated in the margin of the typescript. The drawings should be referred to in the text as 'Text-fig. 1', etc.

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VOLUME 6 *March 1958* PART 1

Contents

MULHERKAR, LEELA
Induction by Regions lateral to the Streak in the Chick Embryo. *With 2 plates* 1

LØVTRUP, SØREN
A Physiological Interpretation of the Mechanism Involved in the Determination of Bilateral Symmetry in Amphibian Embryos 15

RAVEN, CHR. P., ESCHER, FROUKE C. M., HERREBOUT, W. M., *and* LEUSSINK, J. A.
The Formation of the Second Maturation Spindle in the Eggs of *Limnaea*, *Limax*, and *Agriolimax*. *With 2 plates* 28

BILLETT, F. *and* MULHERKAR, LEELA
The Localization of β-Glucuronidase in the Early Chick Embryo. *With 1 plate* 52

MOOG, FLORENCE, *and* LUTWAK-MANN, CECILIA
Observations on Rabbit Blastocysts Prepared as Flat Mounts. *With 1 plate* 57

BELLAIRS, A. D'A.
The Early Development of the Interorbital Septum and the Fate of the Anterior Orbital Cartilages in Birds. *With 1 plate* 68

AMBELLAN, ELISABETH
Comparative Effects of Mono-, Di-, and Triphosphorylated Nucleosides on Amphibian Morphogenesis 86

KATSCH, SEYMOUR *and* BISHOP, D. W.
The Effects of Homologous Testicular and Brain and Heterologous Testicular Homogenates combined with Adjuvant upon the Testes of Guinea-Pigs. *With 1 plate* 94

LYON, MARY F.
Twirler: A Mutant Affecting the Inner Ear of the House Mouse. *With 1 plate* 105

TENCER, R.
Étude autoradiographique de l'incorporation de $^{14}CO_2$ dans des gastrulas d'Axolotl 117

GRÜNEBERG, HANS
Genetical Studies on the Skeleton of the Mouse. XXII. The Development of Danforth's Short-Tail. *With 3 plates* 124

BELLAIRS, RUTH
The Conversion of Yolk into Cytoplasm in the Chick Blastoderm as shown by Electron Microscopy. *With 5 plates* 149

GOMOT, LUCIEN
Interaction ectoderme-mésoderme dans la formation des invaginations uropygiennes des Oiseaux. *Avec deux planches* 162

REY, LOUIS R.
Physiologie du cœur de l'embryon de Poulet *in vitro* après congélation à très basse température 171

LUCAS, D. R. *and* TROWELL, O. A.
In vitro Culture of the Eye and the Retina of the Mouse and Rat. *With 2 plates* 178

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